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by

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ABSTRACT

THE MECHANISMS UNDERLYING COCAINE-INDUCED OVEREXPRESSION OF BASIC FIBROBLAST GROWTH FACTOR (BFGF, FGF2), AN EFFECT REVERSED BY EXTINCTION

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Drug addiction is characterized by compulsive drug use and chronic relapse despite negative consequences. Drug-induced structural and functional changes in the brain are thought to underlie these characteristics. One mechanism that may mediate these characteristics are growth factors, such as basic fibroblast growth factor (bFGF or FGF2), as they are necessary for cellular growth, survival, differentiation, and have roles in memory, mood, and anxiety disorders. bFGF mRNA and protein expression is increased following stimulant administration and is necessary for stimulant-induced changes in dendrites and behavioral sensitization. Moreover, addiction is maintained by cues associated with the drug, as they can can evoke craving and promote relapse. Therefore, reducing cue reactivity, such as with extinction, could reduce relapse rates. Inhibiting bFGF in the infralimbic medial prefrontal cortex (IL-mPFC), following self-administration, facilitates extinction. Extinction of drug seeking can reduce bFGF expression in IL-mPFC, nucleus accumbens (NAc), and dorsal hippocampus (dHipp), indicating that bFGF may mediate drug-associated learning. However, the circuitry and mechanisms underlying extinction and the role of bFGF is unknown. Therefore, the

current experiments investigated if drug-induced plasticity was altered by extinction and if bFGF had a role, in brain regions associated with learning or addiction (i.e., IL-mPFC, NAc, and dHipp). We found that cocaine self-administration induced changes in plasticity-related protein expression, such as ARC and pGSK3β, in each brain region, and extinction could ameliorate some of that plasticity. Moreover, we found that neutralizing bFGF in NAc prior to four 30-min extinction sessions disrupted initial extinction retention. However, if bFGF was neutralized without four 30-min extinction sessions, subsequent extinction was enhanced. In dHipp, neutralizing bFGF, with or without four 30-min extinction sessions, facilitated subsequent extinction over days. Overall, these results suggest that blocking the biological function of bFGF in a number of reward- and learning-related brain regions can facilitate subsequent extinction. Understanding the neuronal mechanisms by which bFGF regulates extinction at systems and molecular levels will allow for development of new pharmacotherapeutics to enhance extinction-based therapies for addiction.

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LIST OF ABBREVIATIONS

ΔFosB: delta FosB

ANOVA: analysis of variance

ARC: activity-regulated cytoskeleton-associated protein

Anti-bFGF: neutralizing antibody against bFGF

β-Actin: beta actin

BDNF: brain derived neurotrophic factor

bFGF: basic fibroblast growth factor; also called fibroblast growth factor 2 (FGF2)

BLA: basolateral amygdala

BSA: bovine serum albumin

Coc: cocaine

CREB: camp response element binding

DSH: disheveled

dHipp: dorsal hippocampus

ERK/MAPK: extracellular-signal-regulated kinase or mitogen-signal regulated kinase

Ext: extinction

FGFR1: fibroblast growth factor receptor 1

FR1: fixed ratio of 1 to 1

GluR1: glutamate receptor 1

Hipp: hippocampus

IEG: immediate early gene

IHC: immunohistochemistry

IL-mPFC: Infralimbic medial prefrontal cortex

IP3: inositol triphosphate

Kal-7: kalirin-7

LA: lateral amygdala

LRP: lipoprotein receptor-related protein

MAP2: microtubule-associated protein 2

MEK: mitogen-activated protein kinase kinase

mPFC: medial prefrontal cortex

NAc: nucleus accumbens

P-β-catenin: phosphorylated beta catenin

P-ERK: phosphorylated ERK

P-GSK-3β: phosphorylated glycogen synthase kinase-3b

PBP: parabrachial pigmented nucleus of VTA; a subregion of VTA

PFC: prefrontal cortex

PI3K: phosphinositide 3-kinase

PLCγ: phospholipase C gamma

PL-mPFC: prelimbic medial prefrontal cortex

PND: postnatal day

PSD95: post-synaptic density 95

ROI: region of interest

SA: self-administration

T-β-catenin: total protein expression of beta catenin

T-GSK-3β: total protein expression of glycogen synthase kinase-3b

vHipp: ventral hippocampus

vmPFC: ventral medial prefrontal cortex

VTA: ventral tegmental area

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INTRODUCTION

Drug addiction is a chronic and persistent disorder (McLellan, Lewis, O'Brien, & Kleber, 2000) that is characterized by compulsive and uncontrollable drug seeking and taking despite negative consequences. Drug use and addiction are highly prevalent, with an estimated 24.6 million people, ages 12 and over, reporting using illegal substances in the United States in 2013 (Substance Abuse and Mental Health Services Administration, 2014). Unfortunately, most treatments are not particularly effective (Conklin & Tiffany, 2002; McLellan et al., 2000), as 30-60 percent of individuals treated for substance abuse return to drug taking within one year of treatment (McLellan et al., 2000). The persistent nature of addiction suggests long-term neuronal changes, and indeed, the changes and mechanisms underlying acute or prolonged drug use, withdrawal, and relapse or reinstatement have been intensely investigated (e.g., Epstein, Preston, Stewart, & Shaham, 2006; Knackstedt & Kalivas, 2009; M. J. Thomas, Kalivas, & Shaham, 2008). In contrast, research concerning neuronal changes underlying treatment is lacking.

A key feature of drug addiction is that it is maintained by cues associated with the rewarding properties of the drug. Following treatment or abstinence, exposure to drug-associated cues can promote craving and withdrawal-like symptoms, which can trigger relapse (Childress, McLellan, & O'Brien, 1986). Therefore, reducing cue-reactivity would reduce relapse rates. One way to reduce reactivity is through extinction. Extinction consists of presenting the previously drug-paired cue in the absence of the drug reinforcer, and following numerous presentations, a new inhibitory memory is formed wherein the cue is no longer associated with the rewarding properties of the

drug (Millan, Marchant, & McNally, 2011; Quirk & Mueller, 2008). The extinction memory is formed in stages, like other forms of learning. First, the new contingencies that the cue no longer predicts the drug are acquired in short-term memory before being subsequently consolidated into long-term storage. On subsequent extinction sessions, the extinction memory can be retrieved, strengthened or weakened, and then reconsolidated back into long-term storage. Extinction-based exposure therapy uses these ideas to treat addiction, however, this type of therapy alone has had limited success (Conklin & Tiffany, 2002). Thus, determining the mechanisms that underlie the persistence of drug addiction, extinction itself, and their interaction, would allow for the development of new pharmacotherapeutics to enhance treatment for drug addiction.

Self-administration paradigm

Drug use and addiction is modeled with rodents using paradigms such as sensitization, classical conditioning, or operant conditioning. Psychomotor sensitization is measured as a progressive increase in locomotor responsiveness to the same dose of a drug, and involves repeatedly injecting an animal with a drug and then measuring their locomotion. Moreover, sensitization is potentiated after a withdrawal period. The psychomotor sensitization paradigm is a frequently used and well validated (Post & Rose, 1976), and is ideal for examining the effects of drug exposure on behavior and the brain (Robinson & Becker, 1986). Sensitization is also long lasting, as it can persist up to months or years (Paulson, Camp, & Robinson, 1991).

Another paradigm commonly utilized uses classical conditioning to train animals to associate one context with drug administration, and another context with vehicle

administration. When the animal is given access to both contexts, they will spend more time in the previously drug-paired chamber, indicating a conditioned place preference (CPP). CPP is frequently used (Bardo & Bevins, 2000), and is ideal for examining a relatively simple drug-associated memory.

To better model prolonged drug use and the complex memories formed by human drug addicts, the self-administration paradigm is used. In this paradigm, animals undergo acquisition of drug taking, during which they are trained to lever press for intravenous infusions of a drug, and these lever presses are often paired with salient cues, such as tones and lights. Throughout training sessions, animals learn to associate the cues with the administration of the drug. Mice, rats, and monkeys will consistently and reliably learn to administer a number of drugs that are commonly abused in humans and will not reliably administer drugs not abused by humans (Koob, 2012; Thomsen & Caine, 2005). Animals lever press for drug for numerous days, allowing for prolonged exposure to the drug and multiple drug-cue pairings, similar to human addicts. Additionally, drug administration is contingent on the animal's behavior, unlike sensitization and CPP in which the drug is administered non-contingently.

Once animals achieve stable drug taking during acquisition, they can be tested under extinction conditions. During extinction, animals are presented with the cue(s), without administration of drug, until lever pressing is substantially reduced. With repeated non-reinforced pairings, the cue(s) becomes associated with the omission of the reinforcer and a new memory is formed. This new memory serves to inhibit drug seeking and the original memory that associates the cues with drug intake. As previously mentioned, the new extinction memory is formed in phases, and each of

these phases can be manipulated (Millan et al., 2011; Quirk & Mueller, 2008). Prior to the first extinction session, acquisition of the new extinction memory can be manipulated, such as with a systemic or intracranial infusion of an agonist or antagonist. Likewise, following the first extinction session, consolidation of the extinction memory can be manipulated.

The initial extinction learning can be further manipulated by shortening the first few extinction sessions. Reducing the time minimizes extinction learning during the first few extinction sessions in order to better manipulate initial learning and determine if the pharmacological manipulation affected the acquisition of the new extinction memory. Retention of the extinction memory is then tested during subsequent full-length extinction sessions (i.e., same amount of time as during acquisition). Poor extinction memory retention is demonstrated by a significant increase in lever pressing from the shortened sessions to the full-length sessions, whereas good extinction memory retention is demonstrated by no change in lever pressing (LaLumiere et al., 2010; Hafenbreidel et al., 2014; Hafenbreidel et al., 2015b). Finally, following extinction, exposing animals to a priming injection of the drug, a stressor, or a non-extinguished drug-associated cue can reinstate lever pressing (Epstein et al., 2006; Lynch, Nicholson, Dance, Morgan, & Foley, 2010). Overall, this paradigm allows for the mechanisms underlying acquisition, withdrawal, extinction, and reinstatement of drug seeking to be examined.

A number of brain regions have been implicated in addiction, extinction, or both, including the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), amygdala,

hippocampus, and ventral tegmental area (VTA; Figure 1; Millan et al., 2011; Quirk & Mueller, 2008).

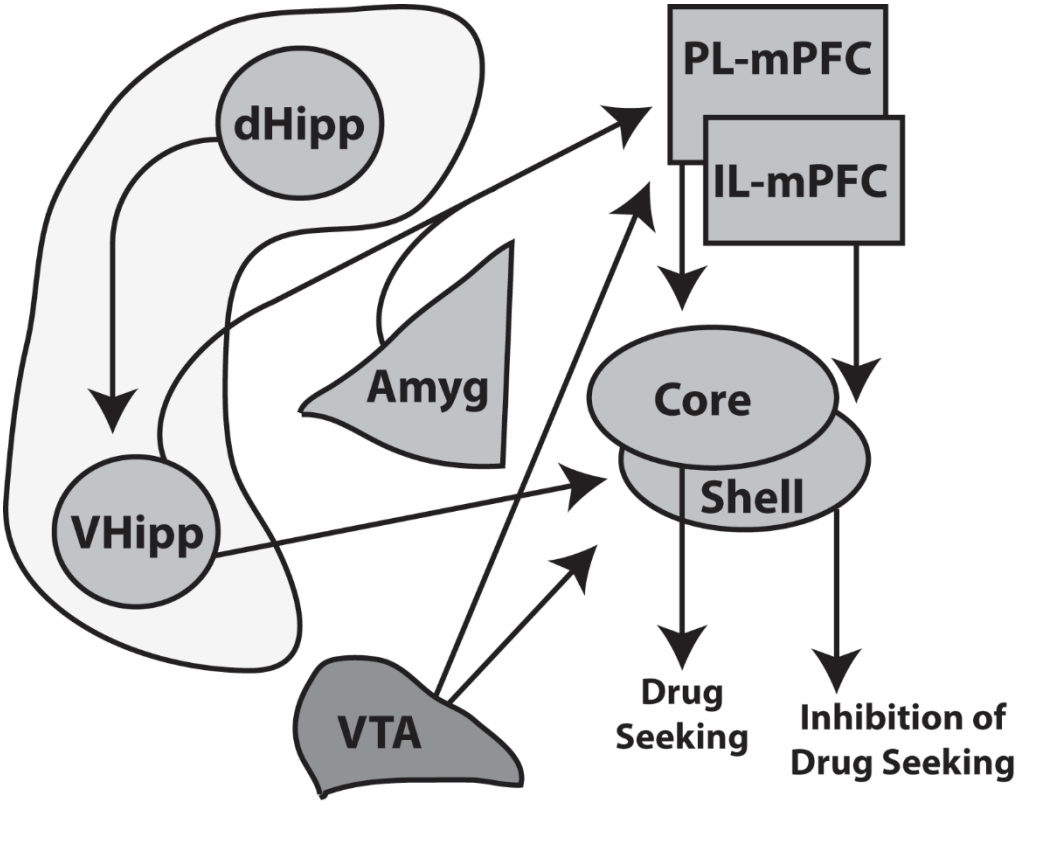

Figure 1: Neural circuitry of drug addiction. A schematic depiction of brain regions and their interactions that are implicated in addiction, extinction, or both (adapted from Otis et al., unpublished); however, the details are still being determined. The medial prefrontal cortex (mPFC; right) is composed of two subregions: the prelimbic (PL) and infralimbic (IL). The PL-mPFC projects to the core region of the nucleus accumbens (NAc) and is thought to drive drug seeking, whereas the IL-mPFC projects to the shell region of the NAc and is thought to inhibit drug seeking following extinction (Millan et al., 2011; Peters, Kalivas, & Quirk, 2009). The ventral tegmental area (VTA) is composed of dopaminergic neurons that project to numerous reward-related brain regions, including the NAc and mPFC (Wise, 2004). The hippocampus (left) is composed of two subregions: the dorsal hippocampus (dHipp) and ventral hippocampus (vHipp). The dHipp indirectly projects to the NAc and mPFC through the vHipp and the amygdala projects to the mPFC, to mediate drug seeking (Fanselow & Dong, 2010; Hiranita, Nawata, Sakimura, Anggadiredja, & Yamamoto, 2006; Kelley, 2004).

Briefly, mPFC is composed of two subregions: the infralimbic mPFC (IL-mPFC) and the prelimbic mPFC (PL-mPFC), which have different roles in addiction and extinction. Inactivating PL-mPFC blocks cocaine- and stress-induced reinstatement (McFarland, Davidge, Lapish, & Kalivas, 2004; McFarland & Kalivas, 2001; McFarland, Lapish, & Kalivas, 2003), and attenuates cue-induced reinstatement (Hiranita et al., 2006; McLaughlin & See, 2003), but has no effect on extinction (LaLumiere, Niehoff, & Kalivas, 2010; Peters, LaLumiere, & Kalivas, 2008). Conversely, inactivating IL-mPFC has no effect on cocaine-, cue-, or stress-induced reinstatement (Hiranita et al., 2006; McFarland et al., 2004; McFarland & Kalivas, 2001; McLaughlin & See, 2003), but will disrupt extinction following self-administration (LaLumiere et al., 2010; Peters et al., 2008), and enhancing IL-mPFC function will facilitate extinction in the self-administration or CPP paradigm (LaLumiere et al., 2010; Gass et al., 2014; Otis et al., 2014a). Moreover, inactivating IL-mPFC after an animal has learned extinction following self-administration will induce reinstatement (Peters et al., 2008). Overall, PL-mPFC is thought to mediate drug seeking, and IL-mPFC mediates inhibition of drug seeking following extinction.

Similar to mPFC, NAc is composed of two functionally different subregions: core and shell. The PL-mPFC and IL-mPFC project to NAc core and NAc shell, respectively (Groenewegen, Galis-de Graaf, & Smeets, 1999; Sesack, Deutch, Roth, & Bunney, 1989). Inactivating NAc core blocks cocaine-, cue-, and stress-induced reinstatement (Hiranita et al., 2006; McFarland et al., 2004; McFarland & Kalivas, 2001). However, inhibiting NAc shell only disrupts stress-induced reinstatement (Hiranita et al., 2006; McFarland et al., 2004; McFarland & Kalivas, 2001), but will reinstate drug seeking if

inactivated after an animal has learned extinction (Peters et al., 2008). Overall, these findings suggest that the connections between PL-mPFC and NAc core drive drug seeking, whereas, the connections between IL-mPFC and NAc shell inhibits drug seeking following extinction (LaLumiere & Kalivas, 2008; McFarland et al., 2004; McFarland et al., 2003; Peters et al., 2009).

A few additional brain regions are often investigated for their role in addiction, including the VTA, amygdala, and hippocampus. The VTA is composed primarily of dopaminergic neurons with wide projections throughout the brain, including PFC and NAc (Aransay, Rodriguez-Lopez, Garcia-Amado, Clasca, & Prensa, 2015), and inactivation of VTA blocks stress-, context-, and cocaine-induced reinstatement (Bossert, Liu, Lu, & Shaham, 2004; McFarland et al., 2004; McFarland & Kalivas, 2001). The amygdala is implicated in the emotional aspects of reinstatement, as inactivating it will block stress- or cue-induced reinstatement (Erb, Salmaso, Rodaros, & Stewart, 2001; Hiranita et al., 2006; Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; Leri, Flores, Rodaros, & Stewart, 2002; McFarland et al., 2004; McLaughlin & See, 2003), but has no effect on cocaine-induced reinstatement (McFarland & Kalivas, 2001) or extinction expression (Peters et al., 2008). Lastly, the hippocampus is composed of the dorsal hippocampus (dHipp) and ventral hippocampus (vHipp), and inactivating either blocks cocaine- and cue-induced restatement (Hiranita et al., 2006).

In summary, a number of paradigms can be used to model drug use and drug-associated learning in rodents, including sensitization, CPP, and self-administration. The self-administration paradigm is specifically used to model complex memories and prolonged drug use in rodents, and can be used to investigate plastic changes that

occur following acquisition, withdrawal, extinction, and reinstatement in a number of reward-related brain regions, including mPFC, NAc, VTA, amygdala, and hippocampus.

Drug-induced plastic changes

Experiences, such as an enriched living environment or a learning event, can induce plastic changes in the brain, such as changes in spine density and neuronal excitability (Kolb & Whishaw, 1998; Sehgal, Song, Ehlers, & Moyer, 2013). These changes can prime the region for subsequent plasticity (W. C. Abraham & Bear, 1996), such as that needed for new learning. Commonly abused drugs, such as cocaine and amphetamine, can also induce plastic changes in the brain. However, instead of priming the region for subsequent plasticity, they hinder new experience-dependent plasticity (Kolb, Gorny, Li, Samaha, & Robinson, 2003). Drug addiction is characterized by preservative use and chronic relapse, and it is thought that the long-lasting changes induced by drug use in reward-related brain regions might underlie these characteristics (Pickens et al., 2011; M. J. Thomas et al., 2008). Here is a brief review of some of the research that has examined morphological, functional, and molecular changes induced by stimulant drug use.

Drug-induced morphological changes

Stimulant drug use can induce long-lasting morphological changes in reward-related brain regions, and these adaptations might underlie long-lasting behavioral changes (Y. Li, Acerbo, & Robinson, 2004). Prolonged injections of cocaine, amphetamine, or nicotine (20-25 days) plus an extended withdrawal period (24 days to

3.5 months) can increase dendritic length and spine density in reward-related brain regions, such as PFC and NAc (Brown & Kolb, 2001; Kolb et al., 2003; Y. Li, Kolb, & Robinson, 2003; Robinson & Kolb, 1997, 1999, 2004). Dendritic length is also increased in dopaminergic neurons in VTA in rats that were briefly treated with amphetamine (three days) during early life followed by seven to 28 days of withdrawal (Mueller, Chapman, & Stewart, 2006). Moreover, the increases in dendritic branches and spine density are not limited to researcher-administered drugs, as they are also observed following cocaine self-administration plus a prolonged withdrawal period (30 days) in NAc shell and PL-mPFC (Robinson, Gorny, Mitton, & Kolb, 2001). Overall, spine and dendritic changes are induced in reward-related brain regions after prolonged stimulant drug use and a withdrawal period.

However, drug-induced plastic changes occur relatively shortly after the cessation of drug use as well. Twenty-four hours after a single injection of cocaine, spine density is increased in VTA (Sarti, Borgland, Kharazia, & Bonci, 2007). Moreover, four or 24 hours after seven days of cocaine injections, thin-type spines in NAc shell are increased, but after 24 hours are decreased in NAc core (Dumitriu et al., 2012). Interestingly, changes in spine density might be dependent on the dose of cocaine given or the context in which the cocaine was given. For example, a low dose of cocaine given in the rats home cage does not induce increased spine density in NAc core, but if that same dose is given in a novel context or is doubled, then spine density is increased in NAc core (Y. Li et al., 2004). Alternatively, when cocaine or methylphenidate (i.e., Ritalin) was administered for a longer period, 15-20 days, dendritic spine density on dopaminergic neurons in NAc core and shell was increased

after only two days of withdrawal (Dobi, Seabold, Christensen, Bock, & Alvarez, 2011; Y. Kim et al., 2009; K. W. Lee et al., 2006). In summary, stimulant drug use can fairly rapidly induce spine changes in reward-related brain regions, however, these changes are contingent on context, dose, and length of time the drug is administered.

In conclusion, stimulant drug use can induce morphological changes in reward-related brain regions. However, the changes are dependent on a number of factors, such as length of drug administration, length of withdrawal, and brain region examined. Overall, dendritic and spine changes are evident following acute and prolonged stimulant administration with a short withdrawal period (Y. Kim et al., 2009; K. W. Lee et al., 2006; Ren et al., 2010; Sarti et al., 2007), following acute stimulant administration with a prolonged withdrawal period (Dobi et al., 2011; Y. Li et al., 2004; Mueller et al., 2006), and following prolonged stimulant administration and withdrawal (Brown & Kolb, 2001; Y. Li et al., 2003; Robinson et al., 2001; Robinson & Kolb, 1997, 1999, 2004). However, the mechanisms underlying the persistence of these changes are still unclear, but they are likely produced by homeostatic mechanisms in response to drug use and withdrawal (Kalivas, 2009; Ren et al., 2010).

Drug-induced functional changes

As discussed, fairly rapid and long-lasting morphological changes in reward-related brain regions are produced by stimulant drug use. These type of changes are not unique to drug use, but instead are often produced following experiences (W. C. Abraham & Bear, 1996; Kolb & Whishaw, 1998), such as living in an enriched environment. However, rats with cocaine experience do not demonstrate increases in

spine density after living in an enriched environment during withdrawal as their drug-naïve counterparts do, indicating that previous drug use blocks new experience-dependent plasticity (Kolb et al., 2003).

Experience-dependent plasticity also occurs following learning (W. C. Abraham & Bear, 1996; Kolb & Whishaw, 1998), but previous drug use can decrease performance on a number of tasks in rodents. For example, two weeks after rats underwent cocaine self-administration, rats had reduced performance on the delayed alternation task using a t-maze (Radley et al., 2015). Moreover, neonatal or early life injections of methamphetamine result in disrupted performance in the Morris water maze during adulthood (Skelton, Williams, Schaefer, & Vorhees, 2007; Vorhees, Ahrens, Acuff-Smith, Schilling, & Fisher, 1994; Williams, Vorhees, Boon, Saber, & Cain, 2002), and binge-like cocaine administration (multiple injections per day across several days) during adolescence (P35-46) results in increased novelty-seeking (or reduced anxiety) in adulthood as measured by time spent in the open arms in the elevated plus maze or in the center of an open field test (Sillivan et al., 2011). Additionally, these rats have attenuated context fear learning, suggesting that previous drug use increased impulsivity, but disrupted new learning (Sillivan et al., 2011). Overall, previous drug use can alter behavior and hinder subsequent learning.

The changes observed in learning and behavior following drug use may be due to changes in neuronal excitability and plasticity. Depending on a number of variables, stimulant drug use can alter synaptic and intrinsic excitability (Kauer & Malenka, 2007; Kourrich, Calu, & Bonci, 2015; Wolf, 2010). For example, synaptic plasticity is enhanced in VTA following a single cocaine injection for at least five days (Ungless,

Whistler, Malenka, & Bonci, 2001), and is enhanced following prolonged cocaine administration (5-28 days) with short or extended withdrawal (2-23 days) in NAc (Dobi et al., 2011; Kourrich, Rothwell, Klug, & Thomas, 2007; Yao et al., 2004) and hippocampus (Guan, Zhang, Xu, & Li, 2009). Synaptic plasticity is enhanced for a protracted amount of time following drug cessation, suggesting enduring homeostatic change. Indeed, glutamatergic AMPA receptors (AMPArs) lacking the GluR2 subunit (GluR2-lacking AMPArs), which are often GluR1-containing AMPArs and have high channel conductance and calcium permeability, are increased in NAc following prolonged withdrawal (Wolf & Ferrario, 2010). This effect is observed progressively following withdrawal from long-access self-administration, which consists of long daily sessions (6+ hours versus 1-2 hours for short-access) or a longer acquisition period (more than the usual 10-14 days for short access; X. Li & Wolf, 2015; Loweth, Tseng, & Wolf, 2014). Moreover, following prolonged withdrawal, animals can demonstrate "incubation" of drug seeking, which is the potentation of reward seeking (Grimm, Fyall, & Osincup, 2005; Grimm, Hope, Wise, & Shaham, 2001; Grimm, Shaham, & Hope, 2002), and is mediated by the increase in GluR2-lacking AMPArs in NAc (Conrad et al., 2008; Wolf & Ferrario, 2010).

Alternatively, intrinsic excitability, or how likely a neuron is to fire an action potential, is decreased following drug administration and withdrawal. Following brief cocaine or amphetamine injections or cocaine self-administration, intrinsic excitability is decreased in NAc (Dong et al., 2006; Ishikawa et al., 2009; Kourrich & Thomas, 2009; Mu et al., 2010) for at least 21 days (Ishikawa et al., 2009). However, activity is increased in NAc following drug-associated cue presentation or drug presentation

following 21-30 days of withdrawal from cocaine self-administration compared to one day of withdrawal (Hollander & Carelli, 2007; Mu et al., 2010). Similar findings are also observed in human addicts. Activity in PFC following short or prolonged withdrawal in human addicts is reduced; however, following drug-associated cue presentation (e.g., videos) activity is significantly increased, which is strongly correlated with self-reported craving (Goldstein & Volkow, 2002, 2011). Overall, these results suggest a decrease in function in NAc and PFC following drug use, but an increase following presentation of drug-associated cues after prolonged withdrawal.

In summary, following drug administration, animals have impaired experience-dependent plasticity, altered behavior, and disrupted new learning. Moreover, rats and humans display decreased neuronal activity in reward-related brain regions following prolonged drug use and withdrawal, but increased activity with drug-associated cue presentation. These behavioral and functional adaptations are likely manifested by drug-induced changes in synaptic and intrinsic excitability, morphology, and molecular components.

Drug-induced molecular changes

As mentioned, structural and functional changes induced by stimulant drug use may also be mediated by molecular changes in drug-related brain regions. Some of these changes have been discussed already, such as alterations in AMPArs. However, numerous other molecules are altered either directly or indirectly following drug use or drug-associated learning, which may mediate the changes discussed previously.

Below, I will highlight a few of the molecular changes induced by chronic drug use (see Table 1 for summary).

One of the most extensively studied addiction-related molecules is the transcription factor delta FosB (ΔFosB), which is a highly stable splice variant of the immediate early gene (IEG) *fosB* (Chen, Kelz, Hope, Nakabeppu, & Nestler, 1997; Hope et al., 1994). ΔFosB expression is increased following administration of cocaine, amphetamine, nicotine, morphine, phencyclidine (PCP), tetrahydrocannabinol (THC), and ethanol in NAc (core and shell), PFC, amygdala, and hippocampus (Atkins et al., 1999; Hiroi et al., 1997; Y. Kim et al., 2009; Larson et al., 2010; K. W. Lee et al., 2006; Nye, Hope, Kelz, Iadarola, & Nestler, 1995; Nye & Nestler, 1996; Perrotti et al., 2008; Pich et al., 1997; Robison & Nestler, 2011; Y. Wang et al., 2015). Mice overexpressing ΔFosB have comparable spine densities in the NAc as mice treated with repeated cocaine, and spine density is further increased when these mice are given repeated cocaine injections (Maze et al., 2010). Furthermore, mice overexpressing ΔFosB have increased cocaine-induced locomotion and enhanced CPP conditioning (Kelz et al., 1999). Moreover, ΔFosB is thought to mediate motivation, as mice overexpressing it have facilitated acquisition of cocaine self-administration at a dose that usually results in poor acquisition, and a higher breaking point with a progressive ratio (Colby, Whisler, Steffen, Nestler, & Self, 2003). In summary, ΔFosB expression is increased in reward-related brain regions following administration of a number of abused drugs, mediates spine density changes, and is thought to mediate the incentive properties of drugs.

	Stimulant drug admin					
Protein	**Acute**	**Chronic**	**Withdrawal**	**Behavioral**	**Extinction**	**Brain region**
bFGF	↑	↑	↑1-30 days	neutralizing bFGF facilitates extinction in lL-mPFC	↓	lL-mPFC, NAc, dHipp, VTA, SNc
GluR1		↑	↑ 21+ days	associated with potentiated seeking	↑	NAc
ΔFosB	↑	↑	↑ 2-14 days	associated with motivation to drug seek		NAc core, NAc shell, PFC, amygdala, hipp
ARC	↑	↑	↑ 3-48 days	retrieval of an associative memory		PL- and lL-mPFC, LA
PSD95	↓	↓	↓ 1-60 days	stimulant-induced sensitization	↑	NAc
MAP2	↑	↓	↑ 14 days, ↓ 7 days		↑	NAc
Kal-7		↑	↑ 1-14 days	associative learning		NAc
pβ-catenin						
tβ-catenin		↓ & ↑	↑ 10 days, ↓ 1 days			NAc, Hipp
pGSK3β	↓	↓	↓ 1 day			NAc, BLA
tGSK3β				stimulant-induced locomotion; drug-associated learning		

Table 1: Summary of stimulant-induced molecular changes. Acute refers to short administration of a stimulant drug (e.g., 1 or 2 injections), and chronic refers to prolonged administration of a stimulant drug (e.g., 14+ days). Up arrows denote an increase in expression of the corresponding protein, down arrows denotes a decrease in expression of the corresponding protein, and blank denotes unknown changes for the correxponding protein. (Alaghband et al., 2014; Atkins et al., 1999; Caffino, Racagni, & Fumagalli, 2011; Flores, Rodaros, & Stewart, 1998; Fumagalli, Pasquale, Racagni, & Riva, 2006; Hafenbreidel, Twining, Rafa Todd, & Mueller, 2015; W. Y. Kim, Jang, Lee, Jang, & Kim, 2013; Kiraly, Ma, et al., 2010; J. S. Miller et al., 2014; Nye & Nestler, 1996; Self, Choi, Simmons, Walker, & Smagula, 2004; Sutton et al., 2003; X. Wang et al., 2013; Wolf & Ferrario, 2010; Xu et al., 2009; Yao et al., 2004).

Another IEG that is associated with stimulant-induced plasticity is activity-regulated cytoskeleton-associated protein (ARC). ARC mRNA and protein expression is increased in the mPFC following a single injection of cocaine in the mPFC, striatum, and hippocampus (Caffino et al., 2011), but not in the VTA or NAc (Rodriguez-Espinosa & Fernandez-Espejo, 2015). These increases in the mPFC are long-lasting as well, as repeated cocaine injections during the adolescence period of a rat (PND28-42) results in increased ARC mRNA expression at PND45 and increased ARC protein expression at PND90 (Caffino et al., 2014; Giannotti et al., 2014). However, ARC expression is not just specifically increased by injections of cocaine. Instead, ARC protein expression is increased in the PL- and IL-mPFC, but not hippocampus, following retrieval of a cocaine conditioned place preference or conditioned activity memory (Alaghband et al., 2014). The increase in ARC expression following memory reactivation has also been demonstrated following retrieval of a conditioned fear memory in the lateral amygdala (Maddox & Schafe, 2011), indicating a specific role for ARC in associative memory retrieval.

Postsynaptic density protein 95 (PSD95) has also been implicated in drug-induced morphological changes. PSD95 is a scaffolding protein associated with AMPAr trafficking (Boudreau & Wolf, 2005; Schnell et al., 2002; Stein, House, Bredt, & Nicoll, 2003), and is decreased in NAc, but not cortex or hippocampus, following 3-5 or 10 days of cocaine administration plus 1-60 days of withdrawal (Yao et al., 2004). These results suggest reorganization of dendritic and spine plasticity, instead of a decrease in spines, which complements other data that found that the occurrence of silent synapses (synapses that lack functional AMPArs) in NAc is increased following cocaine administration (Huang et al., 2009). Moreover, PSD95 knockout mice demonstrate increased responsiveness to a single injection of cocaine, but do not demonstrate sensitization following repeated injections (Yao et al., 2004), suggesting that drug-induced changes in PSD95 may underlie drug-induced behavioral changes.

Another post-synaptic protein, microtubule-associated protein-2 (MAP2), which is associated with dendrites and drendritic growth (Gordon-Weeks, 1991; Shafit-Zagardo & Kalcheva, 1998), has been implicated in drug-induced morphological changes. MAP2 protein expression is increased in the striatum following five days of cocaine administration plus 14 days of withdrawal (Yao et al., 2004), which agrees with previous findings of increased spine density following cocaine administration. However, MAP2 mRNA and protein expression in the NAc is decreased following one week of withdrawal from cocaine self-administration (four hours sessions/12 days) compared to naïve controls, but increased in rats that underwent extinction during the same withdrawal period (Self et al., 2004). These results may indicate reorganization of dendritic and spine plasticity, perhaps specific to withdrawal from self-administration,

similarly to postsynaptic density protein 95 (PSD95) expression following cocaine administration.

Another example is Kalirin-7 (Kal-7), which is a brain-specific guanine-nucleotide exchange factor (GEF) that mediates spine plasticity and excitatory transmission (Carlisle & Kennedy, 2005; Ma et al., 2011; Penzes et al., 2000; Xie et al., 2007), and is localized to the PSD (Kiraly, Eipper-Mains, Mains, & Eipper, 2010; Ma, Wang, Ferraro, Mains, & Eipper, 2008). Repeated cocaine injections increased Kal-7 mRNA and protein expression in NAc (Kiraly, Ma, et al., 2010), which is still elevated for at least 14 days (X. Wang et al., 2013), but these increases are blocked in Kal-7 knockout mice (Kiraly, Ma, et al., 2010). Moreover, shRNA knockdown of Kal-7 blocked cocaine-induced spine and GluR1-containing AMPAr increases in NAc core following withdrawal (X. Wang et al., 2013). Kal-7 knockout mice also have disrupted passive avoidance learning (Kiraly, Lemtiri-Chlieh, Levine, Mains, & Eipper, 2011; Ma, Kiraly, et al., 2008), decreased anxiety in an elevated zero maze (Ma, Kiraly, et al., 2008), disrupted cocaine, but not food, CPP (Kiraly, Ma, et al., 2010), and knockdown in NAc reduced acquisition of cocaine self-administration (X. Wang et al., 2013). In summary, Kal-7 mediates cocaine-induced morphological changes in NAc and mediates anxiety- and drug-related behaviors.

Finally, downstream targets of the Wnt pathway might mediate drug-induced plastic and behavioral changes. Wnt signaling, among other roles (e.g., Fortress & Frick, 2016; Inestrosa & Varela-Nallar, 2014; Maguschak & Ressler, 2012; Oliva, Vargas, & Inestrosa, 2013), is necessary for cell proliferation, differentiation (Esfandiari et al., 2012; Hirabayashi et al., 2004; Kioussi et al., 2002; Zechner et al., 2003), and

dendritic growth and branching (Rosso, Sussman, Wynshaw-Boris, & Salinas, 2005). In the absence of Wnt activation, via binding to its Frizzled receptor, glycogen synthase kinase 3 beta (GSK-3β) phosphorylates β-catenin, tagging it for degradation. However, in the presence of Wnt, GSK-3β is phosphorylated and thus inactivated, allowing β-catenin to translocate to the nucleus and promote gene transcription (Figure 2; MacDonald, Tamai, & He, 2009; Moon, Bowerman, Boutros, & Perrimon, 2002). These targets, GSK-3β and β-catenin, are differentially altered following cocaine administration.

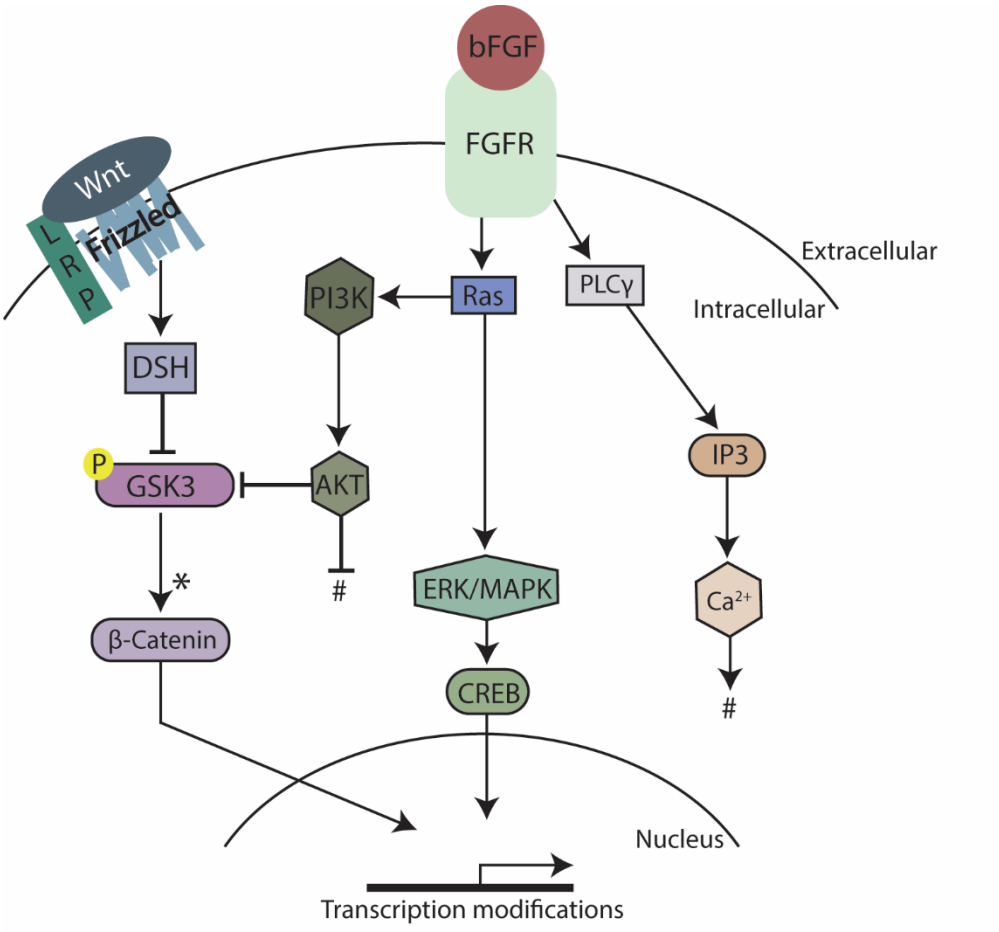

Figure 2: Simplified diagram of bFGF signaling pathway and its interaction with the Wnt signaling pathway. (Far left) Following binding of Wnt to its Frizzled receptor, which includes the association with LRP (lipoprotein receptor-related protein), DSH (disheveled; among others) is activated. This results in

the phosphorylation (p) of GSK-3β (glycogen synthase kinase 3 beta), or its inactivation, resulting in β-catenin not being tagged for degradation, and instead being translocated to the nucleus to modify transcription (represented with a dashed line; Dailey, Ambrosetti, Mansukhani, & Basilico, 2005). (Middle) Following binding of bFGF to its FGFR (fibroblast growth factor receptor), a number of subsequent pathways can be activated, including Ras (Powers, McLeskey, & Wellstein, 2000; Zubilewicz et al., 2001). Ras belongs to a superfamily that mediates a number of downstream proteins and pathways, including the Raf/MEK (mitogen-activated protein kinase kinase)/ERK/MAPK (mitogen/extracellular-signal regulated kinase) pathway, which can activate a number of transcription factors, including CREB (cAMP response element binding; Graham & Richardson, 2011b; Lu, Koya, Zhai, Hope, & Shaham, 2006; Nestler, 2013; Powers et al., 2000; Zubilewicz et al., 2001). Ras also activates the PI3K (phosphinositide 3-kinase)/AKT (also called protein kinase B, PKB) pathway, which can inhibit GSK-3β activity, among a number of other functions (#; Dailey et al., 2005; Peineau et al., 2007; Zubilewicz et al., 2001). (Far right) bFGF binding can also activate PLCγ (phospholipase C gamma; Gu, Seong, Lee, & Kay, 1996), which can subsequently activate IP3 (inositol triphosphate), resulting in modified calcium activity within the cell, as well as mediation of other pathways.

Acute or chronic stimulant drug use enhances GSK-3β activity, as shown by a reduction of GSK-3β phosphorylation at serine 9, in the amygdala (Perrine, Miller, & Unterwald, 2008), NAc core, but not NAc shell, after a relatively short withdrawal period (30 minutes to 24 hours; W. Y. Kim et al., 2013; J. S. Miller et al., 2014; Xu et al., 2011; Xu et al., 2009). Moreover, GSK-3β heterozygous mice, which have a reduction of roughly 50% of GSK-3β in the striatum, have attenuated amphetamine-induced increases in locomotion (Beaulieu et al., 2004); alternatively, pharmacologically blocking GSK-3β phosphorylation in NAc core, resulting in GSK-3β remaining active, increases cocaine-induced locomotion (W. Y. Kim et al., 2013). Similarly, blocking GSK-3β activity systemically or in the NAc core (but not shell) attenuates sensitization development and expression (Enman & Unterwald, 2012; J. S. Miller, Tallarida, & Unterwald, 2009; Xu et al., 2011; Xu et al., 2009). Moreover, the development and later reconsolidation of a cocaine CPP is blocked by inhibiting GSK-3β activity systemically,

in NAc core, or in the basolateral amygdala (J. S. Miller et al., 2014; Shi et al., 2014; Wu et al., 2011). In summary, stimulant drug use increases GSK-3β activity in a number of reward-related brain regions and is necessary for drug-induced increases in locomotion, sensitization, and drug-associative learning.

If cocaine administration results in an increase in GSK-3β activity, then it is likely that β-catenin expression would decrease. In agreement with this, following seven days, but not one day, of cocaine injections plus 24 hours of withdrawal, β-catenin mRNA expression is decreased in NAc (Dias et al., 2015). However, others have reported increased β-catenin mRNA expression in the hippocampus following 14 days of binge-like cocaine administration, 30 minutes after the last injection (Freeman et al., 2001), or in NAc following 10 days of withdrawal, but not after one or 100 days, from cocaine self-administration (Freeman et al., 2010). These discrepancies might be due to differences in cocaine administration, withdrawal length, or due to a lack of subregion specificity in NAc. Overall, more work is needed to determine the role of β-catenin and the Wnt pathway in cocaine-induced plastic and behavioral changes.

In summary, acute or chronic drug administration can have a profound influence on brain morphology, function, and molecular signaling. This influence includes increased spine density and dendritic branching in reward-related brain regions, as well as decreased neuronal activity in these regions following withdrawal, which is reversed and enhanced following drug-associated cue presentation. Moreover, early life drug administration or chronic drug administration can result in disrupted experience-dependent plasticity and new learning in adulthood or after prolonged withdrawal. Lastly, drug use or withdrawal from drug use can modify expression of a number of

plasticity-related molecules, such as ARC, ΔFosB, GluR1, Kal-7, MAP2, PSD95, GSK-3β, and β-catenin. In brief, chronic drug use and subsequent withdrawal can induce structural, functional, and molecular changes in reward-related brain regions. These changes are likely induced as a homeostatic mechanism to regulate the altered neurotransmitter signaling produced by drug use or withdrawal (Kalivas, 2009), however, it is unclear how these molecules are altered following extinction. One potential mechanism underlying these changes might be growth factors, such as basic fibroblast growth factor (bFGF or FGF2).

bFGF and plastic changes

As reviewed, stimulant drug administration can induce morphological, functional, and molecular changes, and one possible mechanism mediating these changes are growth factors, such as bFGF. bFGF is part of a large and functionally diverse fibroblast growth factor family, which is composed of 23 different FGF types and subfamilies (FGF1-23), ten of which are localized to the brain (Burgess & Maciag, 1989; Powers et al., 2000; Reuss & von Bohlen und Halbach, 2003). bFGF has a broad range of functions throughout development and adulthood. For example, during development, bFGF is necessary for cell proliferation, migration, survival, and differentiation (Aoyagi, Nishikawa, Saito, & Abe, 1994; Grothe et al., 2000; Ornitz & Itoh, 2001; Wagner, Black, & DiCicco-Bloom, 1999). Throughout life, bFGF contributes to tissue repair (Schultz & Wysocki, 2009), cancer (Haley & Kim, 2014), and angiogenesis (van Wijk & van Kuppevelt, 2014). Furthermore, bFGF has a role in learning, memory, and mood and anxiety disorders (Riva et al., 2005; Graham and Richardson, 2011a).

bFGF is expressed in both neurons and glia, but preferentially in glia (Eckenstein, Woodward, & Nishi, 1991; Gomez-Pinilla, Lee, & Cotman, 1994; Gonzalez, Berry, Maher, Logan, & Baird, 1995; Reuss & von Bohlen und Halbach, 2003), and a higher molecular weight form of bFGF is specifically expressed in the nucleus, though its function is currently unclear (Arnaud et al., 1999; Bugler, Amalric, & Prats, 1991; Coulier et al., 1997; Powers et al., 2000). Moreover, bFGF is expressed on cell surfaces, in the extracellular matrix, and has extracellular functions (DiMario, Buffinger, Yamada, & Strohman, 1989; Mignatti, Morimoto, & Rifkin, 1992), although it is unclear how bFGF is transported or released to these locations. bFGF lacks a signaling sequence and is not secreted in an endoplasmic reticulum-Golgi pathway dependent manner (J. A. Abraham et al., 1986; D'Amore, 1990; Forget, Stewart, & Trudeau, 2006; Ornitz & Itoh, 2001). Currently, bFGF is thought to be released by cell damage or cell death (Friesel & Maciag, 1999; Mignatti et al., 1992; Miyake et al., 1998; Miyamoto et al., 1993; Ohmachi et al., 2000; Ornitz & Itoh, 2001; Reuss & von Bohlen und Halbach, 2003), though this hypothesis does not explain all the roles attributed to bFGF.

FGFs can bind to four types of FGF receptors (FGFR1-4; D. E. Johnson & Williams, 1993), each with numerous splice variants (Powers et al., 2000), and with different expression patterns. FGFR1 and FGFR4 are preferentially expressed in neurons, FGFR2 is preferentially expressed in oligodendrocytes, and FGFR3 is preferentially expressed in astrocytes (Asai et al., 1993; Miyake, Hattori, Ohta, & Itoh, 1996). bFGF can bind to any FGFRs, but binds with the highest affinity to FGFR1 (P. L. Lee, Johnson, Cousens, Fried, & Williams, 1989; Ornitz et al., 1996; Reuss & von Bohlen und Halbach, 2003). FGFRs are a part of the tyrosine kinase receptor family (D.

E. Johnson & Williams, 1993), and when bound by bFGF are trafficked to the nucleus (Bikfalvi, Klein, Pintucci, & Rifkin, 1997; H. M. Johnson, Subramaniam, Olsnes, & Jans, 2004; Myers, Martins, Ostrowski, & Stachowiak, 2003; X. Zhang & Simons, 2014) where they can alter transcription. However, bound FGFRs also initiate a number of signaling cascades (Dailey et al., 2005), which together mediates a number of plastic changes during development and adulthood (Figure 2).

bFGF and morphological changes during development and in vitro

Growth factors have a major role during embryonic development, and bFGF has been implicated in proliferation, survival, migration, growth, and differentiation (Anderson, Dam, Lee, & Cotman, 1988; Aoyagi et al., 1994; Bikfalvi et al., 1997; Grothe et al., 2000; Wagner et al., 1999). bFGF and FGFR1 protein and mRNA are expressed at high levels at the beginning of neurogenesis in rat and mice embryos, but expression is reduced as neurogenesis and proliferation slows during late embryogenesis (Raballo et al., 2000; Vaccarino et al., 1999). bFGF application to cultured cells increased proliferation in a dose-dependent manner (Boku et al., 2013), and blocking bFGF with a neutralizing antibody (anti-bFGF) reversed these effects (Murdoch & Roskams, 2013; Tao, Black, & DiCicco-Bloom, 1997). Moreover, systemic injection or lateral ventricle infusion of bFGF into newborn rat pups increased proliferation in the hippocampus, pontine subventricular zone, and granule cells in the cerebellum (Tao, Black, & DiCicco-Bloom, 1996; Wagner et al., 1999), and increased mass, volume, and cell number at either E20.5 or 2 months, but the opposite was found when anti-bFGF was infused (Vaccarino et al., 1999) or when bFGF was knocked out (Raballo et al., 2000; Vaccarino

et al., 1999). Overall, these findings indicate that bFGF is necessary for cell proliferation and neurogenesis.

bFGF also interacts with a number cell signaling pathways, such as the Wnt signaling pathway, including GSK-3β and β-catenin, to mediate cell proliferation (Figure 2). bFGF promotes phosphorylation of GSK-3β (Israsena, Hu, Fu, Kan, & Kessler, 2004) through downstream targets, such as AKT (also called protein kinase B, PKB; Dailey et al., 2005), resulting in increased expression of β-catenin in the nucleus and increased cell proliferation (Boku et al., 2013; Israsena et al., 2004; Shimizu et al., 2008). The bFGF-induced increase in proliferation is mediated through GSK-3β, as expressing the dominant-active form of GSK-3β blocks increases in proliferation (Shimizu et al., 2008); whereas, inhibiting GSK-3β with a small molecule increases cell proliferation more so than when bFGF is added alone (Murdoch & Roskams, 2013). Moreover, the increase in proliferation is through the interactions with β-catenin, as there is only enhanced neuronal differentiation and no effect on proliferation in cell culture overexpressing β-catenin but lacking bFGF (Israsena et al., 2004; Shimizu et al., 2008). In summary, bFGF application increase β-catenin expression, as it inhibits GSK-3β, resulting in increased proliferation.

In addition to proliferation and neurogenesis, bFGF mediates axon branching and spine morphology. bFGF application to cultured cortical or hippocampal neurons rapidly increases axon branching compared to control neurons (Kalil, Szebenyi, & Dent, 2000; Patel & McNamara, 1995), and the removal of bFGF results in a reduction of axonal branching to baseline (Aoyagi et al., 1994). Moreover, bFGF increases axonal branching by increasing growth cone size and slowing the advancement of the growth

cone (Szebenyi et al., 2001). During development, bFGF knockout mice have disrupted cellular organization, differentiation, migration, and a 10 percent reduction in overall thickness of the cortex and hippocampus (Dono, Texido, Dussel, Ehmke, & Zeller, 1998). In adulthood, bFGF knockout mice have normal dendritic spine densities in the hippocampus, but have increased spine length (Zechel, Unsicker, & von Bohlen Und Halbach, 2009). Long, immature, spines are associated with behavioral and learning and memory deficits, such as those seen in mice lacking the functional fragile X mental retardation protein, which also display no changes in spine density but an increase in spine length (Ding, Sethna, & Wang, 2014; Grossman, Elisseou, McKinney, & Greenough, 2006). In brief, bFGF mediates spine morphology and axon branching.

In summary, bFGF has numerous functions during embryonic and postnatal development, but one major role is to induce cell proliferation and neurogenesis. bFGF's ability to induce cellular proliferation and neurogenesis persists into adulthood (Monfils et al., 2005; Pieper et al., 2005), and can reverse age-related reductions in neurogenesis (Jin et al., 2003; Kang & Hebert, 2015). bFGF interacts with and stimulates a number of cell signaling pathways, and one interaction that is necessary for proliferation and is affected by drug use (see next section) is its interaction with GSK-3β and β-catenin. Finally, bFGF is necessary for axonal branching and can modify dendritic spine morphology. Stimulant drug administration similarly alters some of these functions, thus, making bFGF a potential underlying mechanism.

bFGF and drug-induced plastic changes

As reviewed, stimulant drugs induce a number of changes in reward-related brain

regions, and these changes may underlie the perseveration of drug addiction. A possible mechanism underlying these changes is growth factors, such as bFGF. As discussed in the previous section, bFGF is necessary for cellular proliferation, neurogenesis, survival, differentiation, and neurite outgrowth. Thus, bFGF might be mediating some of the plastic changes induced by stimulant drug use.

Following stimulant drug administration, bFGF expression is increased in a number of reward-related brain regions. A single systemic injection of nicotine increased bFGF mRNA expression six hours later in the striatum (Maggio et al., 1998), parietal cortex, and entorhinal cortex (Roceri et al., 2001). Drug-induced increases of bFGF are long-lasting as well, as increased bFGF immunoreactivity in VTA and substantia nigra (SNc) was observed following 24 hrs, 72 hrs, one week, and one month after three days of amphetamine injections (Flores et al., 1998). Moreover, increased bFGF mRNA expression is observed following acute or chronic injections of cocaine (one, five, or 14 injections) in PFC and striatum, and in hippocampus in a time-dependent manner following chronic injections (Fumagalli et al., 2006). Likewise, we found a similar increase in bFGF protein expression in IL-mPFC following cocaine self-administration plus 12 days of withdrawal (Hafenbreidel et al., 2015). In sum, a number of stimulant drugs, given acutely or chronically, can produce relatively long-lasting increases in bFGF mRNA or protein expression in a number of brain regions.

The increased expression is likely mediated by drug-induced increases in extracellular neurotransmitters, such dopamine and glutamate (Flores & Stewart, 2000). Administration of a dopamine D1 or D2 receptor antagonist can block nicotine-induced increases in bFGF mRNA expression in the striatum (Roceri et al., 2001), whereas, a

non-selective dopamine agonist or a dopamine D2-selective agonist, but not a D1-selective agonist, can increase bFGF mRNA expression in the striatum (Roceri et al., 2001), PFC and hippocampus (Fumagalli et al., 2003). Likewise, bFGF mRNA expression is increased following glutamate application to cultured cortical astrocytes (Pechan, Chowdhury, Gerdes, & Seifert, 1993), or following a systemic injection of kainate (to induce seizures) in the hippocampus and striatum three and six hours, respectively, after the injection (Riva, Donati, Tascedda, Zolli, & Racagni, 1994; Van Der Wal, Gomez-Pinilla, & Cotman, 1994). Furthermore, amphetamine-induced increases in bFGF immunoreactivity in VTA and SNc are blocked by co-administration of a nonselective glutamate receptor antagonist (Flores et al., 1998) and a competitive nonselective NMDA receptor antagonist (Flores, Samaha, & Stewart, 2000). Overall, increased bFGF expression following stimulant drug use administration is specifically increased following dopamine and glutamate receptor activation.

As mentioned, bFGF is important for cell survival, but is also neuroprotective during development and adulthood (Anderson et al., 1988; Gomez-Pinilla et al., 1992; Logan et al., 1992; Chadi et al., 1993; Mark et al., 1997; Maggio et al., 1998; Flores and Stewart, 2000a), which may underlie the increased expression following drug use (see Figure 3 for overview). Stimulant drug exposure generates a neuroprotective response (Flores & Stewart, 2000). For example, cocaine blocks monoamine reuptake, including dopamine, and the autoxidation of dopamine results in free-radical byproducts (Fornstedt, Pileblad, & Carlsson, 1990). Free radicals can induce oxidative stress and damage cells (Riley, 1994), but also recruit bFGF (Pechan, Chowdhury, & Seifert, 1992), which can protect cells against oxidative stress (Hou, Cohen, & Mytilineou, 1997;

Mark, Keller, Kruman, & Mattson, 1997). The oxidative stress induced by drug use may be a sufficient injury to the cell to release bFGF, as cell injury or damage is thought to be the mechanism by which bFGF is released (Friesel & Maciag, 1999; Mignatti et al., 1992; Miyake et al., 1998; Miyamoto et al., 1993; Ohmachi et al., 2000; Reuss & von Bohlen und Halbach, 2003). In brief, stimulant drug use induces oxidative stress, which results in the recruitment, and perhaps release, of bFGF to protect the cell. However, drug-induced increases in bFGF are long lasting in certain brain regions (Flores et al., 1998; Fumagalli et al., 2006; Hafenbreidel et al., 2015a), and these increases may have additional, possibly aversive, side effects.

The side effects induced by overexpression of bFGF may include some of the long-term changes observed following drug use. For example, bFGF application to cultured hippocampus neurons increases expression of GluR1-containing AMPArs (Cheng et al., 1995). Moreover, amphetamine-induced increases in dendritic length of dopaminergic neurons in VTA are blocked by inhibition of bFGF by anti-bFGF (Mueller et al., 2006). bFGF also mediates behavioral changes induced by drug use as blocking bFGF with anti-bFGF in VTA attenuates amphetamine-induced increases in locomotion and blocks amphetamine-induced sensitization (Flores et al., 2000). Conversely, systemic injections of bFGF on postnatal day (PND) one enhanced initial acquisition of cocaine self-administration during adulthood (Turner et al., 2009). In summary, bFGF contributes to structural and behavioral changes observed following drug use.

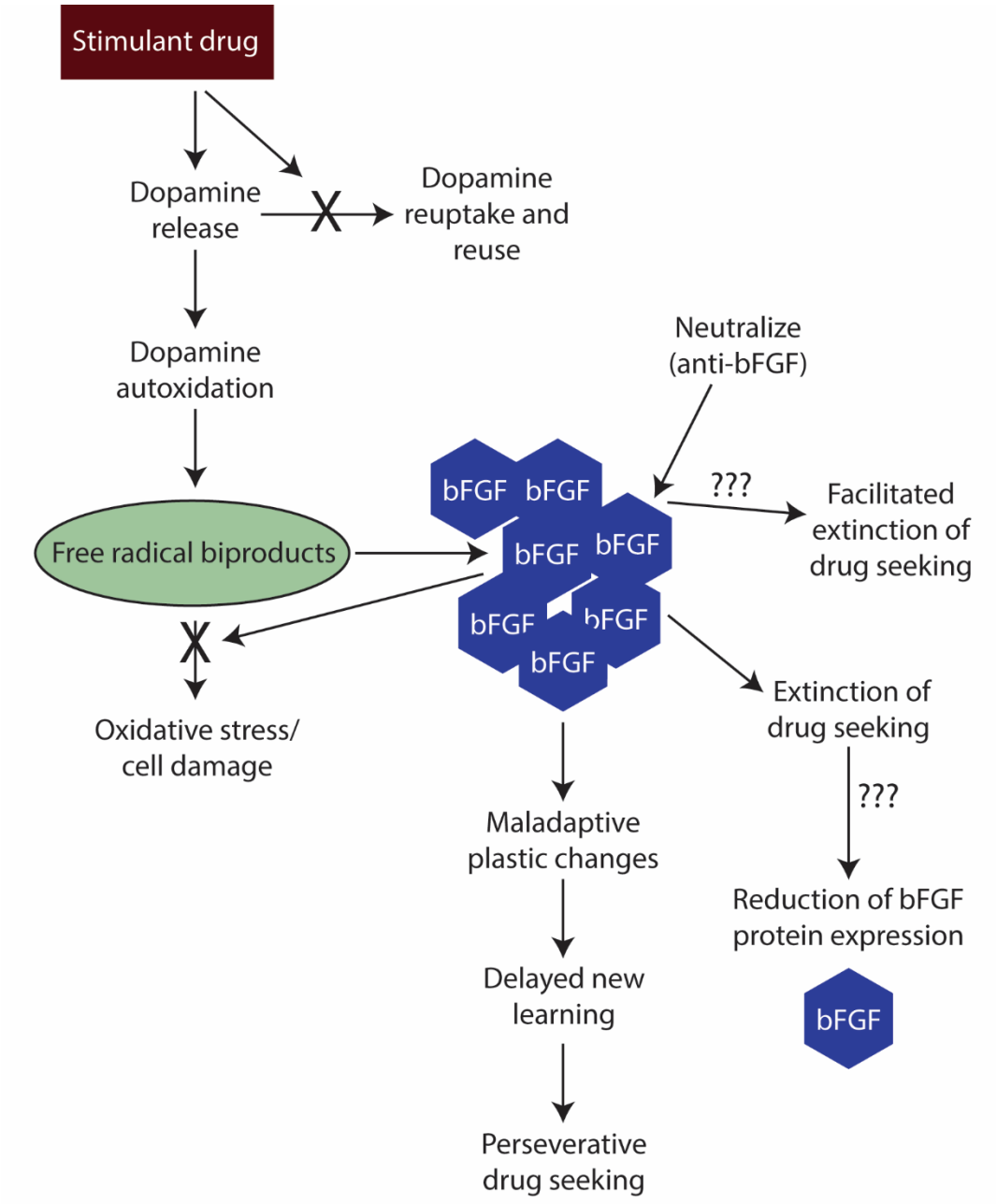

Figure 3: Proposed model of the role of bFGF in drug-associated learning. (Left) Stimulant drug administration blocks dopamine's reuptake, which results in increased extracellular dopamine. Following autooxidaton of dopamine, free radical byproducts are produced (Fornstedt et al., 1990; Kovacic, 2005) that can induce oxidative stress and cell damage (Lopez-Pedrajas et al., 2015; Riley, 1994). Free radicals recruit bFGF (Pechan et al., 1992), which can protect against oxidative stress (Hou et al., 1997; Mark et al., 1997). Drug-induced overexpression of bFGF is long lasting, which can induce a number of maladaptive side effects, including increased GluR1 expression (Cheng et al., 1995), increased dendritic

length (Mueller et al., 2006), decreased excitability (Cuppini, Ambrogini, Lattanzi, Ciuffoli, & Cuppini, 2009; Hilborn, Vaillancourt, & Rane, 1998), and potentiated calcium- dependent inactivation of NMDA receptors (Boxer, Moreno, Rudy, & Ziff, 1999). These changes may underlie the delay in new learning and preservative drug seeking. (Right) We previously found that neutralizing bFGF in the IL-mPFC can facilitate extinction, and extinction can reduce bFGF protein expression (Hafenbreidel et al., 2015). However, it is unclear how neutralizing bFGF facilitates learning, or how extinction can reduce bFGF expression.

Another possible side effect of drug-induced overexpression of bFGF is that it affects neuronal function. Bath application of bFGF inhibits voltage-gated Na+ (Hilborn et al., 1998) and K+ currents, and decreases the number of evoked action potentials in the hippocampus (Cuppini et al., 2009), suggesting that cocaine-induced overexpression of bFGF would functionally reduce intrinsic excitability of neurons. This reduction in intrinsic excitability can have further side effects, as reduced intrinsic excitability limits future learning (Sehgal et al., 2013). Moreover, bFGF inhibits NMDA receptor function following excessive calcium influx (Boxer et al., 1999), which also could impact learning and memory (e.g., McLamb, Williams, Nanry, Wilson, & Tilson, 1990; Mondadori, Weiskrantz, Buerki, Petschke, & Fagg, 1989; Thompson, Winsauer, & Mastropaolo, 1987). In line with this, as discussed previously, stimulant drug use can decrease intrinsic excitability following withdrawal (Kourrich & Thomas, 2009; Mu et al., 2010), hinder later learning (Radley et al., 2015; Sillivan et al., 2011; Skelton et al., 2007; Vorhees et al., 1994; Williams et al., 2002), and limit new experience-dependent plasticity (Kolb et al., 2003). However, if cocaine-induced overexpression of bFGF hinders new learning by inducing maladaptive plastic changes has yet to be directly examined.

In conclusion, bFGF mediates a number of processes during development and adulthood, and in drug-associated behaviors and plasticity. Throughout life, bFGF is neuroprotective, mediates axon, dendrite, and spine morphology, and induces proliferation through its interactions with GSK-3β and β-catenin. However, stimulant drug use results in increased bFGF expression in a number of reward-related brain regions. The initial increase in bFGF expression might be a neuroprotective response, but enduring overexpression may have lasting side effects, such as increased dendritic growth in VTA, reduced intrinsic excitability, and the development and maintenance of drug-associated behaviors, such as locomotor sensitization.

Extinction of drug seeking

As previously mentioned, the persistence of drug addiction is maintained by cues associated with the rewarding properties of the drug (e.g., context or syringe), as these cues can trigger retrieval of the original drug-cue memory, evoke craving, and promote relapse (Epstein et al., 2006; Volkow et al., 2006). Following memory retrieval, the memory is labile and requires restabilization into long-term storage through a process called reconsolidation (C. A. Miller & Marshall, 2005; Misanin, Miller, & Lewis, 1968; Nader, Schafe, & Le Doux, 2000). However, when the cue is repeatedly presented without drug reinforcement, a new inhibitory extinction memory will be acquired and consolidated into long-term storage (Millan et al., 2011; Quirk & Mueller, 2008). Therefore, cue-reactivity can be reduced through extinction, leading to a reduced likelihood of inducing craving and relapse.

As discussed, bFGF mRNA and protein expression is increased following stimulant drug administration (Flores et al., 1998; Fumagalli et al., 2006; Maggio et al.,

1998; Roceri et al., 2001) or cocaine self-administration (Hafenbreidel et al., 2015) in reward-related brain regions, such as IL-mPFC and striatum. Interestingly, we found that extinction of drug seeking following cocaine self-administration reduces bFGF protein expression in IL-mPFC to similar expression levels as controls (Hafenbreidel et al., 2015), and our preliminary data suggests that extinction reduces bFGF expression in dHipp, and possibly in NAc, as well. The reduction in bFGF following extinction is functional as well, as blocking bFGF in IL-mPFC with anti-bFGF facilitates extinction retention following cocaine self-administration (see preliminary data; Hafenbreidel et al., 2015). Although not directly tested, these results suggest that drug-induced overexpression of bFGF impedes new learning, such as that required for extinction.

However, these effects are likely limited to extinction of drug seeking, as bFGF administered systemically or infused into the amygdala enhances extinction of conditioned fear, attenuates renewal following extinction, and attenuates shock-induced reinstatement (Graham and Richardson, 2009a, 2010a, 2011b, a). Moreover, systemic injections of bFGF enhances context fear conditioning in young rats (Graham & Richardson, 2009), and early life systemic injections of bFGF (PND1-5) can enhance later life context fear conditioning and renewal following extinction (PND16) in rats that otherwise demonstrate poor learning at this age (Graham & Richardson, 2010). Overall, these results suggest that bFGF administration enhances extinction of conditioned fear, but early life administration enhances later life fear-associated learning.

Alternatively, bFGF also has a role in anxiety-related behaviors. For example, increased bFGF expression is associated with reduced anxiety-related behaviors and

vice versa, in selectively bred ratlines (Graham & Richardson, 2011b; Riva, Molteni, Bedogni, Racagni, & Fumagalli, 2005). Thus, it is unclear if bFGF simply enhances learning in other paradigms, or if it has a unique and differential role in fear conditioning and drug addiction. It seems likely that bFGF has a unique role in fear- and drug-associated learning, as a systemic injection of bFGF on PND one had no effect on Morris water maze learning or food self-administration (Turner et al., 2009). However, Graham and Richardson (2009b, 2010b) also found a single injection on PND one was not sufficient to induce enhance learning at a young age, but when the number of injections was increased to five and fear conditioning parameters were altered (e.g., more trials, stronger shock) then early life bFGF administration was sufficient to enhance learning. Further experiments are needed to determine the overall role of bFGF in learning and memory, particularly the differential effects of bFGF in fear conditioning and drug-related learning.

The discrepancy in the role of bFGF during extinction of conditioned fear versus extinction of drug seeking is likely due to changes produced by stimulant drug administration on bFGF expression (Flores et al., 1998; Fumagalli et al., 2006; Hafenbreidel et al., 2015; Maggio et al., 1998; Roceri et al., 2001). As discussed in the previous section, this includes increased expression likely from drug-induced increases in extracellular neurotransmitters, such as dopamine and glutamate, and from the neuroprotective response initiated by drug use (Pechan et al., 1992; Pechan et al., 1993; Riva et al., 1994; Mark et al., 1997; Flores et al., 2000; Flores and Stewart, 2000a; Roceri et al., 2001; Fumagalli et al., 2003). In further support of this, we found that bFGF protein expression in IL-mPFC is unchanged following sucrose self-

administration and yoked-saline administration, with or without extinction, compared to naïve home-cage controls (see preliminary data; Hafenbreidel et al., 2015). These results indicate that changes in bFGF expression are specifically due to cocaine administration and subsequent extinction, and not due to operant responding, food reward, or cue presentation. Therefore, the differences in the role of bFGF observed between extinction of drug seeking and extinction of conditioned fear are likely due to the maladaptive changes induced by chronic stimulant drug administration. However, this has not been directly examined, and it is unclear how bFGF mediates these drug-induced changes, or how extinction affects them.

Surprisingly little research has been conducted on the mechanisms underlying extinction of drug seeking, as most research focuses on the development or acquisition of drug seeking, withdrawal, or relapse or reinstatement of drug seeking. Nonetheless, the mechanisms underlying extinction of drug seeking are beginning to be examined. For example, spine diameter in NAc core is increased in rats that underwent extinction of drug seeking following cocaine self-administration compared to yoked-saline controls (Shen, Gipson, Huits, & Kalivas, 2014). Moreover, spine density was increased in IL-mPFC in rats that underwent extinction of drug seeking in an ethanol self-administration paradigm compared to rats that just underwent withdrawal (Gass et al., 2014).

Similarly, the molecular mechanisms underlying extinction have been minimally examined. As mentioned before, ΔFosB, Kal-7, GSK-3β, or β-catenin are all modified following cocaine use, however, it is currently unclear whether extinction modifies their expression. Nevertheless, ΔFosB does have a role in extinction, as overexpression in NAc shell facilitates extinction following cocaine self-administration (Y. Zhang et al.,

2014). It seems likely that their expression would change following extinction, as they are altered or necessary in other learning paradigms, such as classical conditioning or spatial learning (e.g., Fortress, Schram, Tuscher, & Frick, 2013; Greenwood et al., 2011; Kimura et al., 2008; Kiraly et al., 2011; Mandela & Ma, 2012; Mills et al., 2014; Werme et al., 2002).

Changes in glutamatergic receptors and their associated scaffolding proteins (e.g., PSD95) have been measured following extinction. For example, extinction of drug seeking following cocaine self-administration results in increased expression of GluR1-containing AMPAr in NAc shell, however, expression is not changed following withdrawal of a similar passage of time (Ghasemzadeh et al., 2009b), after one day of withdrawal, or after extinction of sucrose seeking (Self et al., 2004; Sutton et al., 2003). Moreover, the increase in GluR1-containing AMPArs is associated with the length of extinction training that occurred; in other words, the better rats learned extinction, the higher their expression of GluR1-containing AMPArs (Sutton et al., 2003). Likewise, PSD95 protein expression is increased in NAc core following extinction after long-access cocaine self-administration compared to saline self-administration, however, no changes were found in NAc shell, dorsomedial PFC (dmPFC, containing PL-mPFC), ventromedial PFC (vmPFC, containing IL-mPFC), or VTA (Ghasemzadeh et al., 2009a; Ghasemzadeh et al., 2009b; Ghasemzadeh et al., 2011). Additionally, PSD95 is increased in PSD subfractions in NAc core following extinction after short-access cocaine self-administration compared to yoked-saline controls (Knackstedt et al., 2010). Changes were specific to extinguishing drug seeking, as PSD95 was not altered following extinction of sucrose seeking or after only two days of extinction training

(Knackstedt et al., 2010). In summary, these results indicate that additional changes occur following extinction that are different than those that occur following withdrawal from drug use. Understanding these changes further could help establish new methods of strengthening extinction, and thus reduce relapse rates.

Despite the progress made regarding how stimulant drug use induces morphological, molecular, and functional changes in a number of reward-related brain regions, and how bFGF mediates morphological and functional changes during development, adulthood, and following stimulant drug administration; it is unclear how these two overlap, and in which brain regions, during extinction of drug seeking. Specifically, little is known concerning plastic changes following extinction of drug seeking and what mechanisms underlie our previous findings that bFGF is reduced following extinction and reducing bFGF facilitates extinction retention. Therefore, this dissertation aims to address these unknowns.

PROPOSED EXPERIMENTS

The proposed experiments aimed to address four central questions: 1) are cocaine-induced plastic changes altered by extinction, 2) in which brain regions do these effects occur, 3) does neutralizing bFGF in the IL-mPFC prior to four 30-min extinction sessions facilitate any extinction-induced plastic changes, and 4) what is the functional relevance of the observed changes in bFGF expression in the NAc and dHipp. Here, I reviewed previous work demonstrating that stimulant drug use can induce a number of plastic changes, and growth factors, such as bFGF, might underlie these changes. I also reviewed the limited data demonstrating that extinction might be able to reverse some drug-induce plastic changes.

bFGF mRNA and protein expression is increased in a number of reward-related brain regions (Flores et al., 1998; Fumagalli et al., 2006; Hafenbreidel et al., 2015; Maggio et al., 1998; Roceri et al., 2001), but decreased following extinction (Hafenbreidel et al., 2015), suggesting a role in both drug-induced plasticity and during extinction. The drug-induced overexpression of bFGF might be a neuroprotective response, as stimulant drugs increase extracellular dopamine that when it undergoes autoxidation, it releases free radical byproducts (Fornstedt et al., 1990; Kovacic, 2005). Free radical byproducts can induce oxidative stress (Lopez-Pedrajas et al., 2015; Riley, 1994), but they also recruit bFGF (Pechan et al., 1992), which can protect against the oxidative stress (Hou et al., 1997; Mark et al., 1997).

The recruited bFGF, however, is long lasting, as it is still observed a long as a month following drug administration (Flores et al., 1998; Fumagalli et al., 2006). These long-lasting increases correspond with plastic and behavioral changes; such as,

increased dendrite length in VTA following amphetamine administration (Mueller et al., 2006), and bFGF in VTA is necessary for cocaine sensitization (Flores et al., 2000). In summary, drug-induced and long-lasting overexpression of bFGF could inadvertently lead to additional plastic changes that promote preservative drug seeking and delay new learning. However, our preliminary data suggests that extinction can reduce bFGF expression in IL-mPFC, dHipp, and possibly NAc, and that blocking bFGF in IL-mPFC can facilitate extinction retention. The behavioral relevance of the reduction of bFGF in dHipp and possibly NAc has yet to be determined. Moreover, if extinction can reverse drug-induced plasticity is not clear.

One limitation to our previous findings is that bFGF was neutralized in IL-mPFC following a 36-38 day withdrawal period. Conversely, we measured bFGF protein expression after a relatively short withdrawal period (12 days) following cocaine self-administration. As reviewed, prolonged withdrawal can induce a number of behavioral and plastic changes in reward-related brain regions (Divac, 1971; Lu, Grimm, Hope, & Shaham, 2004; Radley et al., 2015; Robinson & Becker, 1986; Robinson & Kolb, 1997, 1999, 2004; Sillivan et al., 2011; Skelton et al., 2007; Vorhees et al., 1994; Williams et al., 2002). However, if bFGF expression is altered following prolonged withdrawal is unclear. Moreover, our preliminary data suggests that extinction of drug seeking can alter bFGF expression in a number of brain regions, but the behavioral relevance of these changes are unknown.

Given previous work and our preliminary findings, it is likely that maladaptive plastic and functional changes induced by drug administration are mediated, in part, by bFGF. Extinction then reduces bFGF expression, resulting in the reversal of

downstream consequences of stimulant-induced overexpression of bFGF and stimulant-induced plastic changes. However, one side effect of cocaine-induced overexpression of bFGF is a delay in new learning, as demonstrated by the numerous extinction sessions needed to reach extinction criteria and the facilitation of extinction when bFGF is neutralized.

Aim one: To determine if extinction modifies cocaine-induced plastic changes, and if bFGF mediates these changes.

Stimulant drug administration can induced a number of structural, functional, and molecular changes, such as increased spine density and length, and altered spine morphology, in reward-related brain regions (e.g., Mueller et al., 2006; Robinson & Kolb, 1997, 1999). Furthermore, extinction of drug seeking can induce plastic changes, such as increased spine density in IL-mPFC following extinction of ethanol seeking (Gass et al., 2014). However, if extinction of drug seeking alters stimulant-induced plasticity, and if bFGF mediates these changes are unclear. Based on our preliminary data, the working hypothesis for this aim is that extinction reverses cocaine-induced plasticity, in part, by a reduction in bFGF in three reward related brain regions: IL-mPFC, NAc, and dHipp.

Aim two: To determine the functional relevance of extinction-induced reduction of bFGF in dHipp and NAc.

Both dHipp and NAc have been implicated in extinction. For example, lesioning dHipp disrupts extinction of sucrose seeking (Dillon, Qu, Marcus, & Dodart, 2008) indicating dHipp is necessary for acquiring the new extinction memory. Additionally, inhibiting NAc activity following extinction of drug seeking in a self-administration

paradigm will reinstate drug seeking, suggesting the NAc is necessary for the expression of extinction of drug seeking (Peters et al., 2008). Our preliminary data suggests that extinction of drug seeking following cocaine self-administration decreases bFGF expression in dHipp and possibly NAc. Similarly to our findings in IL-mPFC, the working hypothesis for this aim is that neutralizing bFGF in dHipp or NAc will facilitate extinction retention.

SIGNIFICANCE OF THE PROPOSED EXPERIMENTS

Our previous findings, along with others, suggest that stimulant drug use results in a maladaptive overexpression of bFGF, which promotes plastic changes that may underlie the persistence of drug addiction. The completion of these experiments would determine if extinction can alter cocaine-induced plastic changes, and if these changes are mediated by bFGF. Moreover, they would determine the underlying circuitry mediating our preliminary results. Specifically, these experiments would determine if neutralizing bFGF in other brain regions, such as NAc and dHipp, are also necessary to facilitate extinction retention or if IL-mPFC is unique. Therefore, the behavioral relevance of the observed reduction of bFGF expression in dHipp and NAc following extinction will be determined (see preliminary data). Overall, understanding the neuronal mechanisms by which bFGF mediates extinction at systems and molecular levels would allow for development of new pharmacotherapeutics to enhance extinction-based therapies for addiction.

PRELIMINARY DATA

As described, bFGF mRNA and protein expression is increased following single or multiple injections of cocaine, nicotine, or amphetamine (Flores et al., 1998; Fumagalli et al., 2006; Maggio et al., 1998; Roceri et al., 2001). However, if bFGF protein expression was altered following self-administration or extinction, and if bFGF had a functional role during extinction of drug seeking was unknown. Therefore, we previously conducted a serious of experiments to assess these unknowns. Tissue was collected following an extinction retention test from a number of groups and brain regions (Table 2), and the results demonstrate that cocaine self-administration and extinction can alter bFGF protein expression. Furthermore, we found that blocking bFGF in IL-mPFC facilitated extinction of drug seeking (Hafenbreidel et al., 2015).

Experiment	Treatment	Method	Tissue collected
1	Suc-ext Coc-ext Coc-noext	WB	vmPFC & NAc
2	Naïve Suc-ext Suc-noext Y-sal-ext Y-sal-noext Coc-ext Coc-noext	WB	IL, NAc, & dHipp
3	Naïve Coc-ext Coc-noext	IHC	mPFC, NAc, Amygdala, Hipp, VTA

Table 2: List of experimental groups and tissue collected for each experiment that utilized western blots (WB) or immunohistochemistry (IHC).

Cocaine self-administration increases bFGF protein expression in IL-mPFC and NAc, an effect reversed by extinction

To determine if bFGF was similarly increased following cocaine self-administration and if extinction of drug seeking had an affect on its expression, bFGF protein expression was first measured in vmPFC and NAc after withdrawal or extinction following cocaine self-administration. Rats were trained to lever press for cocaine or sucrose before being matched and assigned to groups: extinction (coc-ext; suc-ext) or no extinction (coc-noext; experiment 1, Table 2 & 3). Rats then underwent extinction of sucrose seeking (Figure 4a) or cocaine seeking (Figure 4b), during which the rest of the rats remained in their home cages. Following the last day of extinction, all rats were tested for extinction retention (Figure 5c), before rats were euthanized and vmPFC and NAc were collected for western blot analysis (see Materials and Methods).

Experiment	Treatment	Active lever	Inactive lever	Infusions
1	Suc-ext (10)	80.13 ± 6.53	-	-
	Coc-noext (7)	39.33 ± 5.97	1.24 ± 0.57	27.05 ± 2.64
	Coc-ext (7)	30.29 ± 4.39	3.81 ± 3.48	25.38 ± 2.89
2	Naïve (7)	-	-	-
	Suc-noext (8)	88 ± 4.23	-	-
	Suc-ext (9)	87 ± 5.56	-	-
	Y-sal-noext (12)	6.89 ± 1.93	3.14 ± 0.71	-
	Y-sal-ext (11)	5.24 ± 1.72	3.55 ± 1.58	-
	Coc-noext (12)	37.83 ± 4.08	4.94 ± 3.11	27.56 ± 1.90
	Coc-ext (12)	39.81 ± 4.21	0.56 ± 0.23	28.22 ± 1.45
3	Naïve (14)	-	-	-
	Coc-noext (9)	39.22 ± 2.64	1.30 ± 0.78	30.15 ± 1.06
	Coc-ext (9)	41.74 ± 3.35	0.78 ± 0.33	29.48 ± 1.02
4	Veh (7)	38.19 ± 4.97	8.10 ± 4.86	26.90 ± 1.56
	Anti-bFGF (8)	36.75 ± 2.77	8.08 ± 2.96	27.86 ± 0.99
5	Veh (10)	43.13 ± 3.59	1.83 ± 0.63	29.90 ± 1.29
	Anti-bFGF (10)	45.03 ± 9.31	9.60 ± 8.61	28.40 ± 1.49

Table 3: Average number of active and inactive lever presses, or infusions across the last three days of cocaine self-administration, for each experiment. There were no significant differences between groups. Numbers in parentheses are final group sizes.

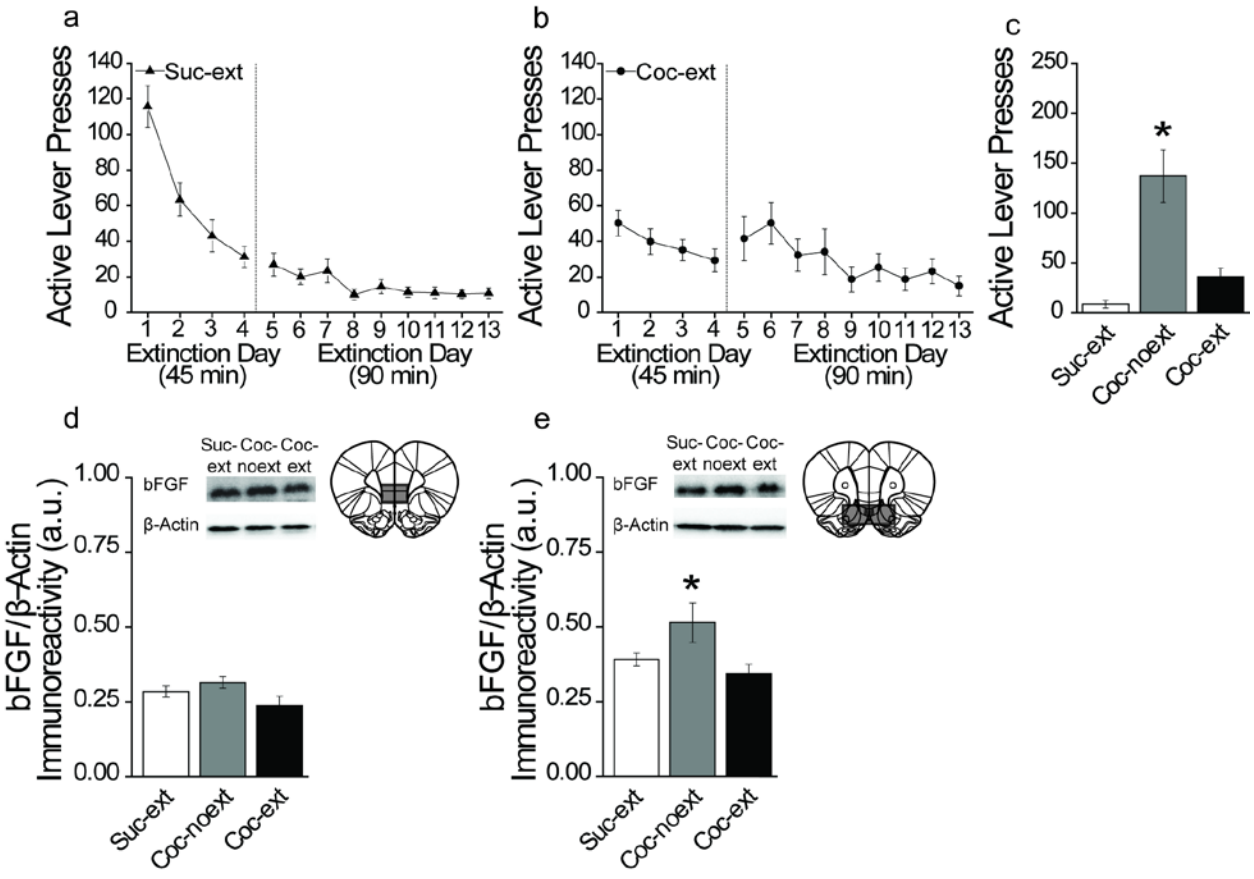

Figure 4: Cocaine self-administration increases bFGF protein expression in NAc, an effect reversed by extinction. Active lever presses during extinction of sucrose seeking (a), cocaine seeking (b), and during the extinction retention test (c). (d-e, top) Representative blots for each group and tissue regions containing the vmPFC or NAc. (d) bFGF protein expression in the vmPFC was not significantly different between groups, but there was a trend between coc-ext and coc-noext groups (t_{12}=2.118, p=0.056). (e) bFGF protein expression in the NAc was greater in coc-noext rats compared to coc-ext or suc-ext rats. Error bars are ±SEM. *p<0.05. Suc-ext, n=10; coc-noext, n=7; coc-ext, n=7.

In vmPFC, bFGF protein expression was not significantly different between groups, but there was a trend for reduced bFGF protein expression in coc-ext rats compared to coc-noext rats (Figure 4d). However, in NAc, bFGF protein expression

was significantly increased in coc-noext rats, replicating previous findings (Fumagalli et al., 2006; Maggio et al., 1998; Roceri et al., 2001), but expression was reversed to baseline following extinction (Figure 4e). Additionally, extracellular-signal-regulated kinase (ERK; also called mitogen-signal regulated kinase, MAPK) and the activated or phosphorylated version of ERK (pERK) were measured, as it is a downstream target of bFGF (Figure 2; Dailey et al., 2005). Moreover, ERK is activated following acute or prolonged cocaine administration (Valjent et al., 2000), is necessary for the development of cocaine or amphetamine sensitization (Valjent et al., 2006b), is increased in sensitized rats (Boudreau, Reimers, Milovanovic, & Wolf, 2007), and is necessary for drug-associated learning (Lu et al., 2006; C. A. Miller & Marshall, 2005; Valjent, Corbille, Bertran-Gonzalez, Herve, & Girault, 2006). Nonetheless, no significant differences between groups were observed in vmPFC or NAc (data not shown), possibly because rats had undergone withdrawal or extinction for over two weeks. Differences might be detectable following the last day of self-administration or following the first day of extinction (e.g., Ren et al., 2010). In summary, these results demonstrated for the first time that extinction reverses cocaine-induced increases in bFGF protein expression in NAc, and were trending in vmPFC.

However, this first preliminary experiment had a number of limitations. Therefore, it was replicated with a number of changes, including the inclusion of additional control groups (experiment 2, Table 1; Hafenbreidel et al., 2015), specifically measuring protein expression in IL-mPFC, and also measuring expression of bFGF primary receptor, fibroblast growth factor receptor-1 (FGFR1; P. L. Lee et al., 1989; Reuss & von Bohlen und Halbach, 2003). Rats underwent sucrose or cocaine self-

administration or yoked-saline administration, in which rats were yoked to a cocaine-administering rat throughout acquisition and extinction (if applicable), receiving identical cues and a saline infusion when the cocaine-administering rat pressed the active lever, but their lever presses resulted in no programmed consequences. A naïve home-cage control group was also included (naïve; experiment 2, Table 2). Following acquisition (experiment 2, Table 3), half of each group underwent extinction (Figure 5a, 5c, & 5e), whereas the other half of each group remained in their home cages. Following the last day of extinction, all rats were tested for extinction retention (Figure 5b, 5d, & 5f), before rats were euthanized and IL-mPFC was collected for western blot analysis. NAc and dHipp were also collected, but not analyzed.

In IL-mPFC, bFGF protein expression was not significantly different between naïve, suc-noext, and suc-ext groups (Figure 6c), indicating that neither sucrose reinforcement nor extinction of sucrose reinforcement altered bFGF protein expression. Furthermore, naïve, yoked-sal-noext, and yoked-sal-ext groups were not significantly different (Figure 6d inset), indicating that cue presentation and i.v. infusions did not affect bFGF protein expression. Therefore, these groups were collapsed into a single control group and compared to coc-noext and coc-ext groups (Figure 6d), and only cocaine self-administration increased bFGF protein expression in IL-mPFC, which was reversed to baseline by extinction. Conversely, FGFR1 protein expression was not significantly different between any groups (Figure 6e, 6f inset, 6f). In summary, these first two experiments indicate that bFGF protein expression was increased following cocaine self-administration in the NAc and IL-mPFC, an effect reversed by extinction, but FGFR1 protein expression in IL-mPFC was not changed in any condition.

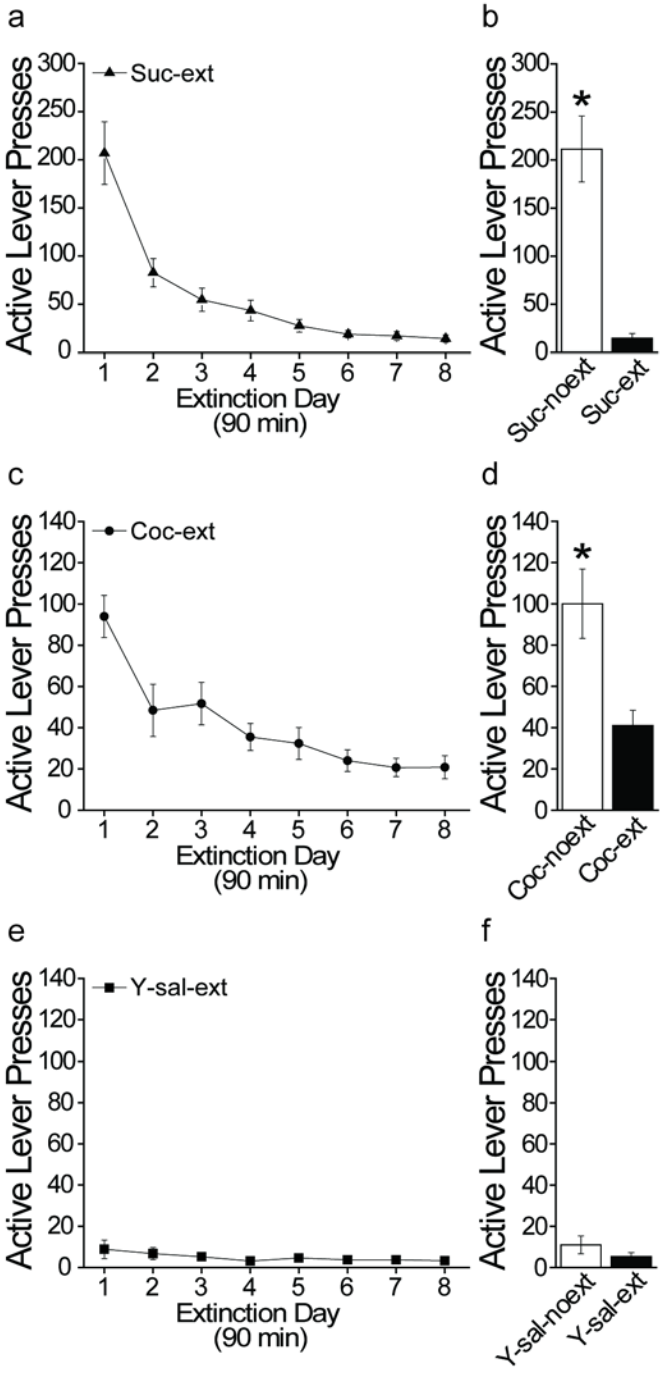

Figure 5: Extinction and extinction retention tests prior to protein analysis. Active lever presses during extinction of sucrose seeking (a) and during the extinction retention test (b). Active lever presses during extinction of cocaine seeking (c) and during the extinction retention test (d). Active lever presses during extinction following yoked-saline (e) and during the extinction retention test (f). Error bars are ±SEM. *p<0.05. Suc-noext, n=8; suc-ext, n=9; yoked-saline-noext, n=12; yoked-saline-ext, n=11; coc-noext, n=12; coc-ext, n=12.

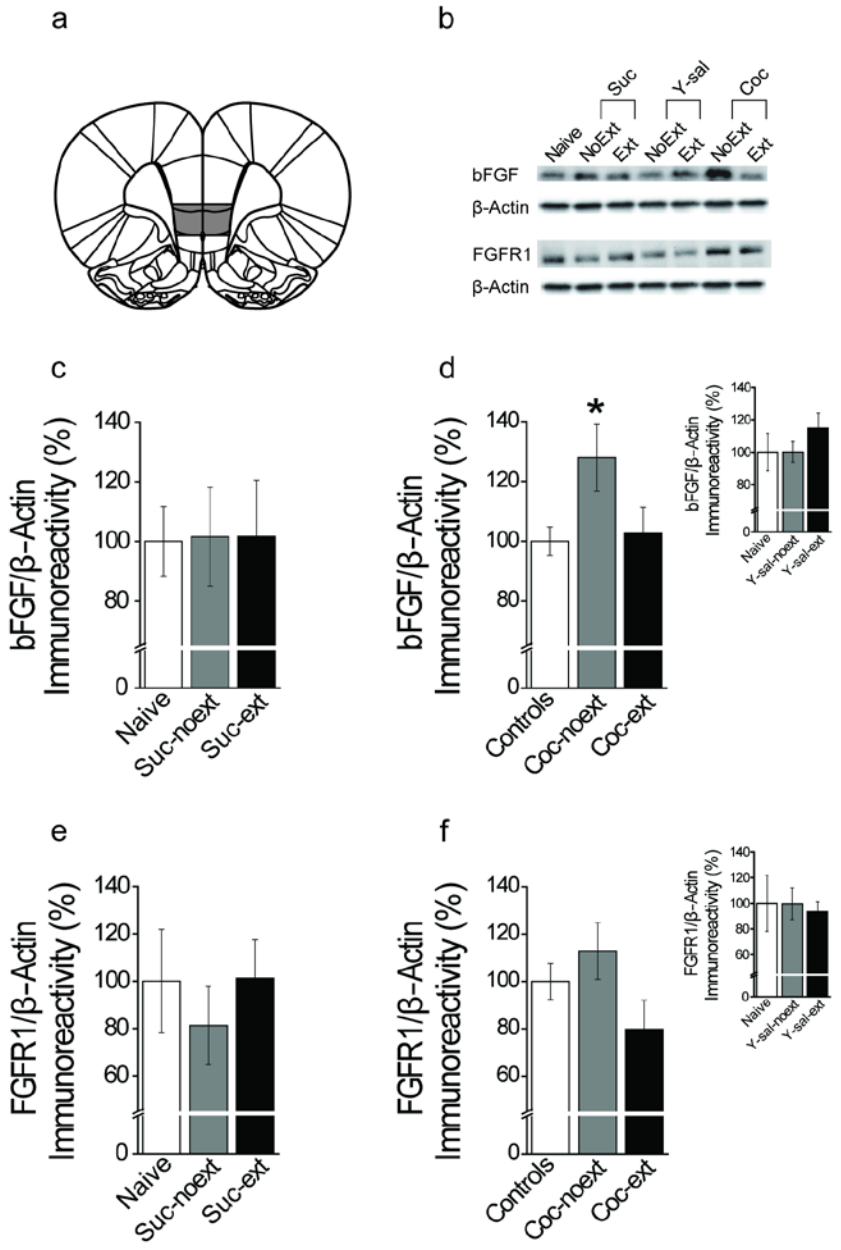

Figure 6: Cocaine self-administration increases bFGF protein expression in IL-mPFC, an effect reversed by extinction. (a) Representative areas of tissue collection containing IL-mPFC. (b) Representative blots for each group. (c) bFGF protein expression was not altered between naïve (n=7), suc-noext (n=8), or suc-ext (n=8) groups. (d) bFGF protein expression was greater in coc-noext rats (n=12) compared to coc-ext rats (n=12) and controls (n=30). (d, inset) Controls are shown separately. bFGF protein expression was not altered between naïve (n=7), yoked-sal-noext (n=12), or yoked-sal-ext (n=11) groups, and these groups were collapsed into a single control group. (e) FGFR1 protein expression was not altered between naïve (n=7), suc-noext (n=7), or suc-ext (n=8) groups. (f) FGFR1 protein expression was not altered between controls (n=28), coc-noext (n=11), or coc-ext (n=11) groups. (f, inset) Controls are show separately. FGFR1 protein expression was not altered between naïve (n=7), yoked-sal-noext (n=11), or yoked-sal-ext groups (n=10), and these groups were collapsed into a single control group. *p<0.05. Error bars are ±SEM.

Cocaine self-administration and extinction of drug seeking alters bFGF expression in NAc and hippocampus

To further examine bFGF protein expression following cocaine self-administration and extinction, bFGF immunoreactivity was measured in a number of brain regions using immunohistochemistry (IHC) as it allows for precise regional specificity and cellular expression, instead of total protein concentration as measured with western blots. Rats were trained to lever press for cocaine before being matched and assigned to groups: extinction (coc-ext) or no extinction (coc-noext). Additionally, a naïve home-cage control group was included (naïve; experiment 3, Table 2 & 3). Rats then underwent daily extinction sessions (Figure 7a) or remained in their home cages. Following the last extinction day, all rats were tested for extinction retention (7b), before being euthanized and brains were collected for IHC processing and analysis (Figure 8 and 9). The brain regions examined were selected because they are important in addiction, learning and memory, extinction, or in combination (see Self-administration paradigm; Fanselow & Dong, 2010; Millan et al., 2011; Quirk & Mueller, 2008), and included PL-mPFC, IL-mPFC, NAc core, NAc shell, amygdala, dHipp, vHipp, and VTA.

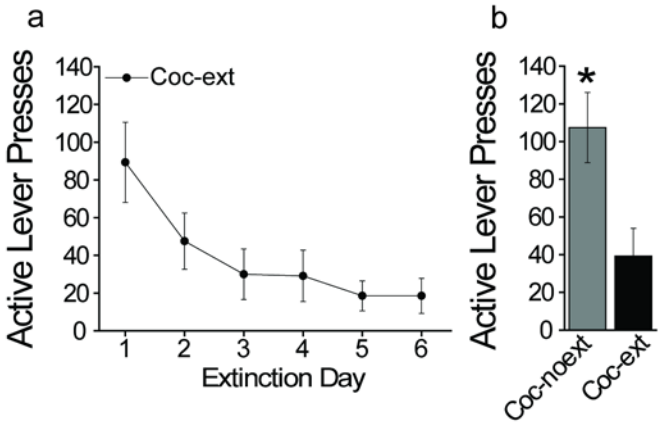

Figure 7: Extinction and extinction retention tests prior to immunohistochemical analysis. Active lever presses during extinction of cocaine seeking (a) and during the extinction retention test (b). *p<0.05 Error bars are ±SEM.

Rats were overdosed with pentobarbital and perfused with phosphate buffered saline and paraformaldehyde. Next, slices from each treatment group that contained mPFC, NAc, amygdala, dHipp, vHipp, and VTA were collected and processed with IHC to measure cells expressing bFGF. Three slices were randomly selected from each rat, imaged, and coded by a non-involved individual to ensure researchers were blind to treatment. Next, regions of interest (ROIs) were drawn for each structure on each slice, followed by semi-manual counting of cells expressing bFGF. After all images were counted, images were decoded, densities per ROIs were calculated (number of cells divided by ROI area in mm^2), and averaged per rat, per treatment group.

bFGF immunoreactivity was not significantly different between groups in PL-mPFC (Figure 8e, left) or in IL-mPFC (Figure 8e, right). However, in IL-mPFC there was a trend for reduced bFGF immunoreactivity in coc-ext rats compared to naïve, suggesting that bFGF immunoreactivity is reduced following extinction. In NAc core (Figure 8g, right), bFGF immnoreactivity was significantly higher in coc-ext rats compared to coc-noext rats, indicating an increase in bFGF immunoreactivity following extinction. However, in NAc shell, there were no significant differences between groups (Figure 8g, left), but there was a trend for a reduction in coc-noext rats compared to naïve rats and coc-ext rats, suggesting that extinction normalizes bFGF immunoreactivity. Overall, no differences were observed in PL-mPFC, but there was a trend in IL-mPFC that extinction reduced bFGF expression. Alternatively, in NAc core and trending in the shell, extinction from drug seeking appears to increase bFGF immnoreactivity compared to undergoing withdrawal.

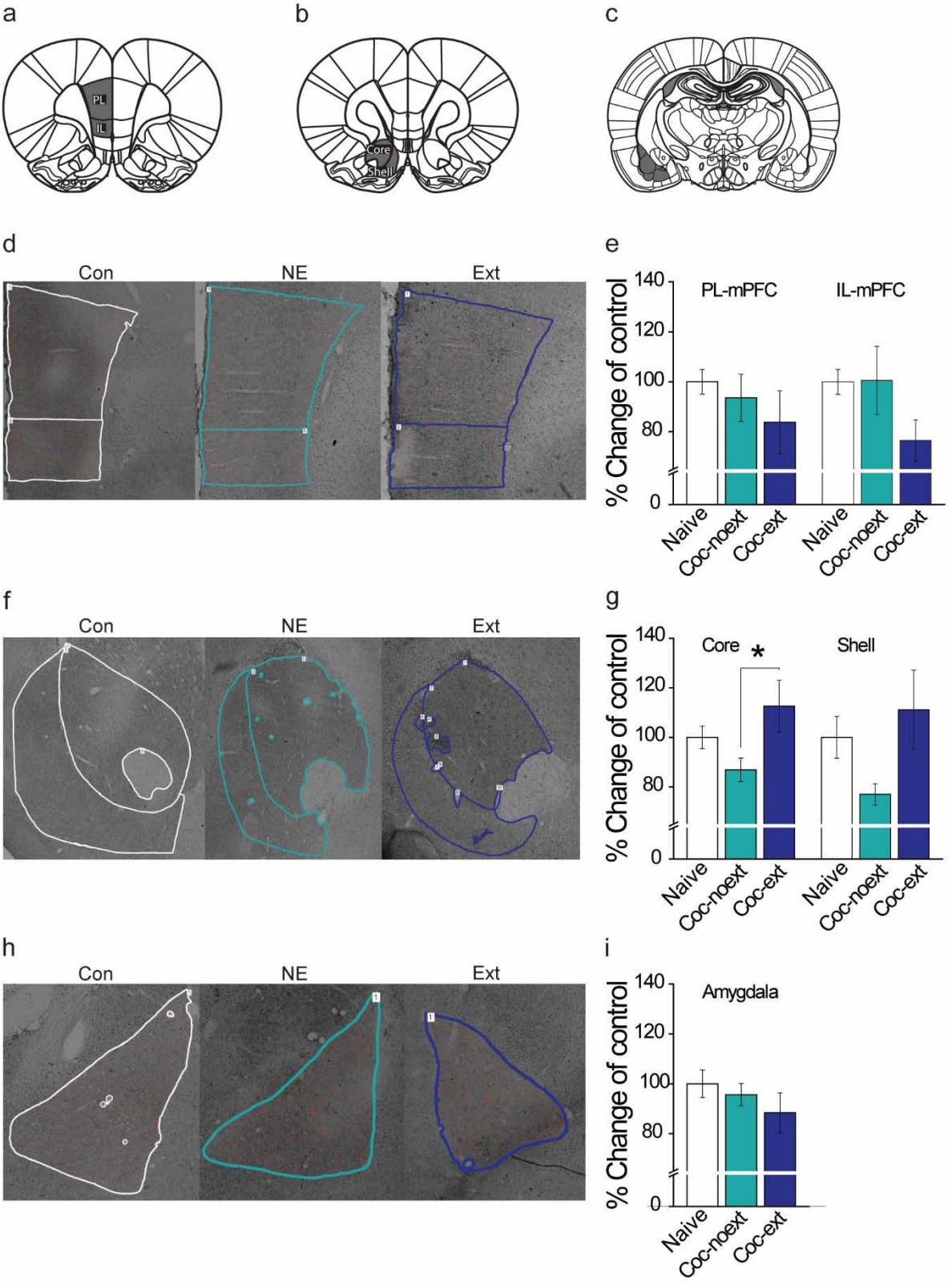

Figure 8: Cocaine self-administration decreased bFGF immunoreactivity in NAc core, an effect reversed by extinction. Representative region of interest (ROI) analyzed, and representative image from each

group, from PL-mPFC and IL-mPFC (a, d), NAc core and shell (b, f), and amygdala (c, h). (e) bFGF immunoreactivity was not significantly different between groups in the PL-mPFC (naïve, n=6; coc-noext, n=5; coc-ext, n=4) or IL-mPFC (naïve, n= 7; coc-noext, n=5; coc-ext, n=4). (g) bFGF immunoreactivity in the NAc was increased in coc-ext (n=4) compared to coc-noext (n=6) rats, but was not different than naïve (n=7) rats. There were no differences between groups in the NAc shell, but there was a trend ($F_{2,14}$=3.319, p=0.066; naïve, n= 7; coc-noext, n=6; coc-ext, n=4). (i) bFGF immunoreactivity was not significantly different between groups in the amygdala (naïve, n= 11; coc-noext, n=7; coc-ext, n=7). *p<0.05. Error bars are ±SEM.

Unfortunately, due to procedural limitations, group sizes and tissue quality likely limited our analysis of these forebrain regions. The trends observed in IL-mPFC moderately match our previous findings that bFGF protein expression is reduced following extinction (Hafenbreidel et al., 2015). However, the increase in bFGF following withdrawal (coc-noext) was not observed when examining cell immunoreactivity. Interestingly, the significant findings and trends observed in NAc core and shell, respectively, are opposite of previous results. Using western blots, bFGF protein expression was increased following withdrawal, an effect reversed by extinction; however, using IHC, there was a reduction in bFGF immnoreactivity following withdrawal (coc-noext), but this was returned to control levels following extinction. These differences may be due to small group sizes and poor tissue quality, or they may be due to examining cellular expression of bFGF with more precisely defined brain regions versus total protein expression in larger, less precisely defined, brain regions. Further experiments are needed to determine how bFGF expression changes in these brain regions, specifically in NAc, following self-administration and extinction.

bFGF expression was also examined in the amygdala, hippocampus, VTA, and PBP (parabrachial pigmented nucleus of VTA; a subregion of VTA). In the amygdala, bFGF immnoreactivity was not significantly different between groups (Figure 9i). In

dHipp, bFGF immnoreactivity was significantly lower in coc-ext rats compared to naïve and coc-noext rats (Figure 9e), demonstrating a reduction in bFGF expression following extinction, which agrees with previous findings in IL-mPFC and NAc with western blots. Alternatively, in vHipp, bFGF immunoreactivity was significantly lower in coc-noext rats compared to naïve (Figure 9g), suggesting that cocaine self-administration reduces bFGF immnoreactivity in vHipp, but extinction mitigates this effect. Finally, bFGF immunoreactivity was not significant different between groups in the VTA (Figure 9i, left) or PBP (Figure 9i, right).

The dHipp and vHipp are thought to regulate different functions, with dHipp mediating cognitive processes associated with learning, memory, and spatial navigation, and vHipp mediating processes associated with emotions and motivation (Fanselow & Dong, 2010). Withdrawal from cocaine is associated with depression and increased anxiety (Aston-Jones & Harris, 2004; Chartoff & Carlezon, 2014; Erb, 2010; Gawin & Kleber, 1986; Koob, 2009), therefore vHipp might be mediating these affective processes during acquisition and withdrawal. However, further investigation is needed to determine the role of bFGF in the hippocampus following cocaine self-administrating and extinction.

Alternatively, bFGF immnoreactivity was unchanged in PL-mPFC, amygdala, and VTA. Results in PL-mPFC may also be limited by procedures, but PL-mPFC and amygdala are necessary for stress- or cue-induced reinstatement (Erb et al., 2001; Hiranita et al., 2006; Kantak et al., 2002; Leri et al., 2002; McFarland et al., 2004; McFarland & Kalivas, 2001; McLaughlin & See, 2003; Peters et al., 2008), therefore, changes in bFGF immnoreactivity might be detectable following reinstatement.

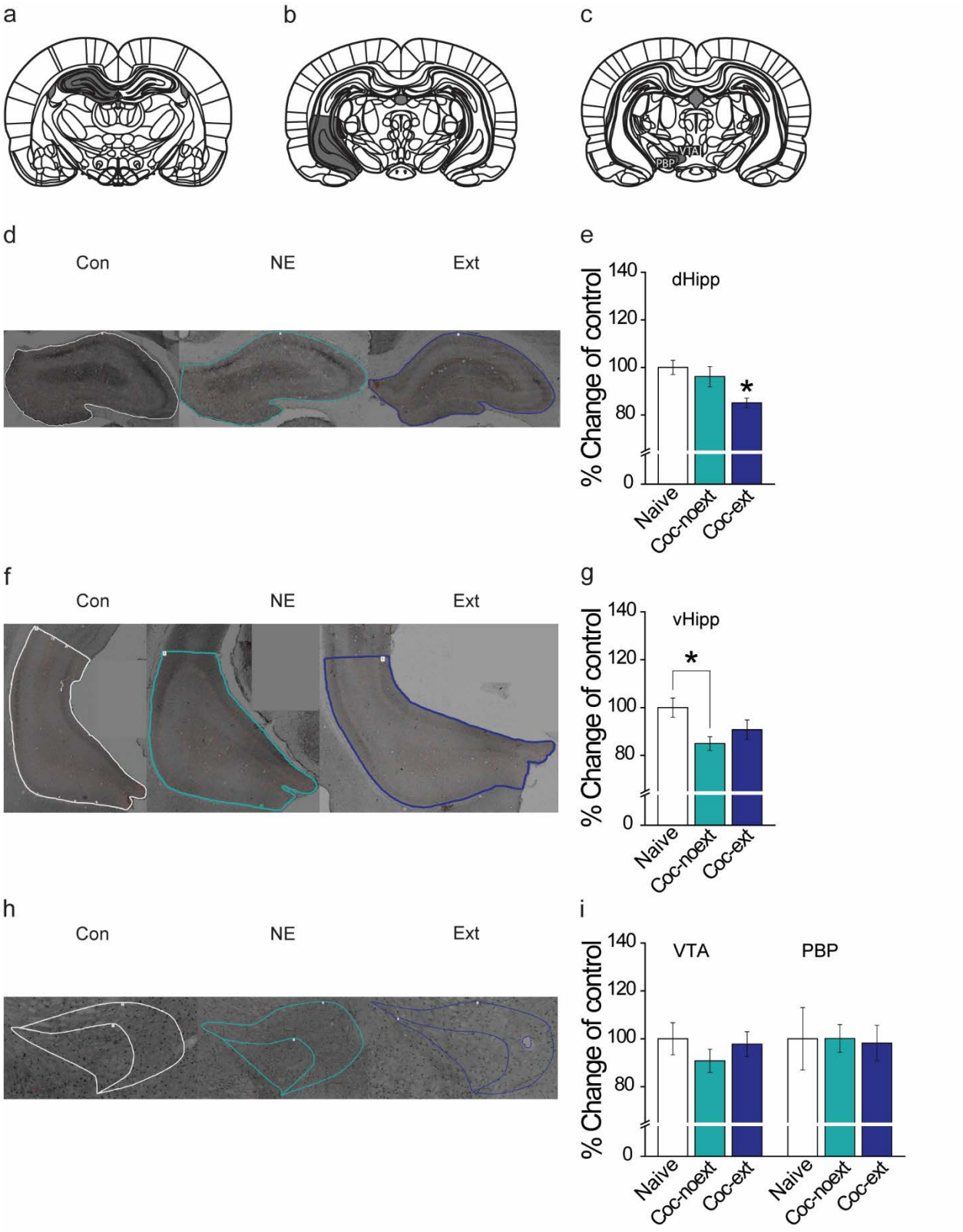

Figure 9: Cocaine self-administration and extinction differentially affects bFGF immunoreactivity in dHipp and vHipp. Representative ROI analyzed, and representative image from each group, from dHipp (a, d),

vHipp (b, f), and VTA (c, h). (e) bFGF immunoreactivity in dHipp was significantly reduced in coc-ext rats (n=9) compared to coc-noext (n=9) and naïve (n=13) rats. (g) bFGF immunoreactivity in vHipp was reduced in coc-noext (n=9) compared to naïve (n=14) rats, but not compared to coc-ext rats (n=9). (i) bFGF immunoreactivity was not significantly different between groups in VTA (naïve, n= 7; coc-noext, n=7; coc-ext, n=6) or PBP (naïve, n= 7; coc-noext, n=7; coc-ext, n=6). *p<0.05. Error bars are ±SEM.

Moreover, VTA is necessary for the development of sensitization (Kalivas & Weber, 1988; Perugini & Vezina, 1994; Vezina & Stewart, 1990), but not for the maintenance or longevity of sensitization (X. F. Zhang, Hu, White, & Wolf, 1997). Similarly, changes in bFGF immnoreactivity in the VTA might have been observed had expression been measured during initial acquisition.

In summary, these results demonstrate that cocaine self-administration and extinction can influence bFGF expression in a number of reward-related brain regions. Significant changes were observed in NAc core, dHipp, and vHipp, but not in mPFC, NAc shell, amygdala, or VTA. Unfortunately, procedural limitations might have limited the results in the mPFC and NAc, but overall, these results suggest subsequent structures to examine for the role of bFGF in drug addiction and extinction learning.

Neutralizing bFGF in IL-mPFC facilitates extinction of drug seeking

Our previous findings indicate that bFGF protein expression in the IL-mPFC is increased following cocaine self-administration, and extinction can reverse this effect. To directly test the function of bFGF during extinction of drug seeking following self-administration, rats were infused with vehicle or anti-bFGF prior to four 30-min extinction sessions and retention was tested on subsequent 90-min extinction sessions. Rats were first trained to self-administer cocaine, before being matched and assigned to

groups: vehicle (veh) or anti-bFGF (experiment 4, Table 2 & 3). Daily extinction training began 38 days after the last day of cocaine self-administration to maximize drug seeking through prolonged withdrawal (as opposed to three days with all previous preliminary experiments; Pickens et al., 2011). Overall, little extinction was observed across the first four 30-min extinction sessions (Figure 10b, left), but anti-bFGF-infused rats showed a robust reduction in lever pressing during the first 90-min extinction session compared to vehicle-infused rats (Figure 10b, right). These results indicate that both groups demonstrated extinction of lever pressing across days, but that neutralizing bFGF in IL-mPFC prior to four 30-min sessions significantly facilitated extinction compared to controls.

Furthermore, a control experiment was conducted to determine if a reduction in bFGF in the IL-mPFC was sufficient to reduce drug seeking, or if an interaction between extinction learning and reduced bFGF was necessary. Rats were trained to self-administer cocaine before being matched and assigned to groups: veh or anti-bFGF (experiment 5, Table 2 and 3). Daily extinction training began 36 days after the last day of cocaine self-administration. Rats were then infused for four days with either vehicle or anti-bFGF into IL-mPFC, and then immediately placed back in their home cages. Overall, both groups extinguished across the 90-min extinction sessions, with no differences between groups (Figure 10e, right). Thus, neutralizing bFGF in IL-mPFC without extinction training (immediately returning rats to their home cages) did not affect subsequent extinction. Therefore, extinction training in combination with bFGF neutralization in IL-mPFC is necessary to facilitate extinction.

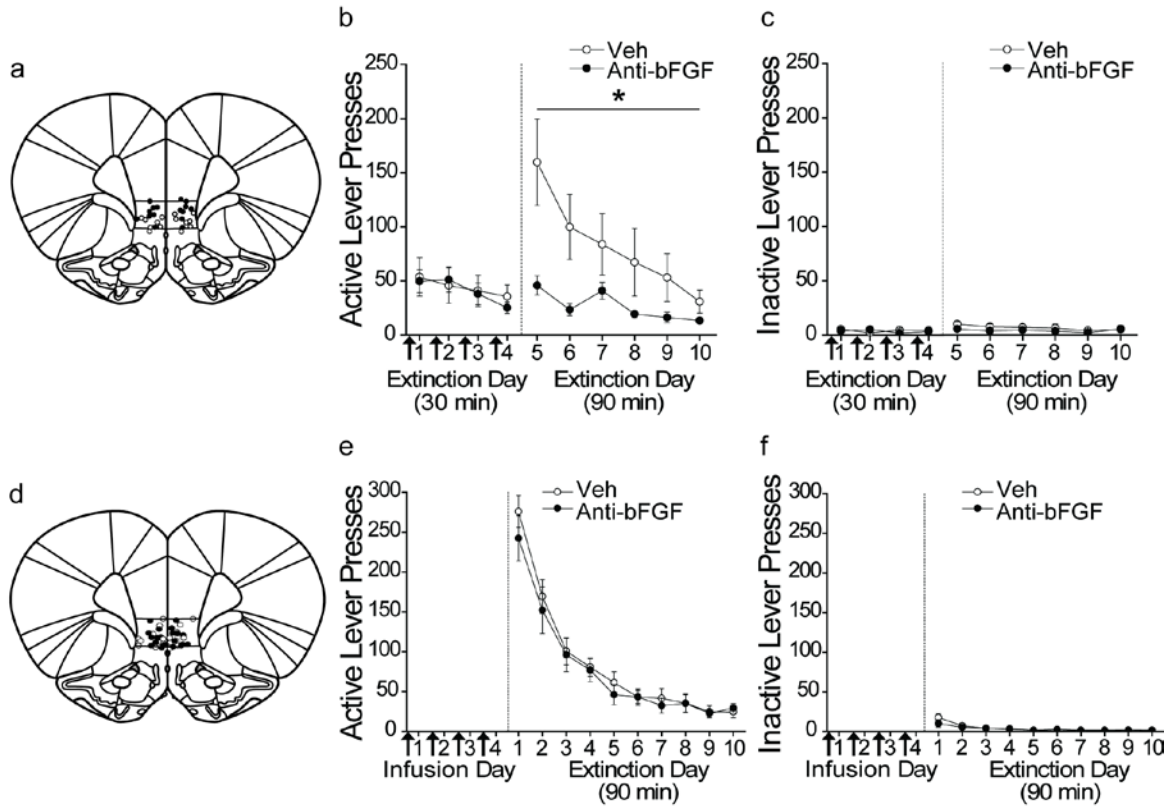

Figure 10: Neutralizing bFGF in IL-mPFC facilitates extinction. (a) Injector tip locations for vehicle (open circles; n=7) and anti-bFGF (closed circles; n=8) in IL-mPFC. (b) Pre-extinction session infusions of anti-bFGF into IL-mPFC (arrows) during the 30-min extinction sessions facilitated extinction retention compared to vehicle infusions. (c) Inactive lever presses during extinction. (d) Injector tip locations for vehicle (open circles; n=10) and anti-bFGF (closed circles; n=10) in IL-mPFC. (e) Infusions of either vehicle or anti-bFGF into IL-mPFC for four days (arrows), during which rats were returned immediately to their home cages, did not affect subsequent extinction. (f) Inactive lever presses during extinction. *p<0.05 Error bars are ±SEM.

MATERIALS AND METHODS

Aim one: To determine if extinction modifies cocaine-induced plastic changes, and if bFGF mediates these changes.

Subjects

Male Long-Evans rats (Harlan) weighing 250-300g at the start of the experiment were individually housed and handled on a 14/10 hour light/dark cycle as previously described (Hafenbreidel, Rafa Todd, Twining, Tuscher, & Mueller, 2014). Rats had unlimited water access, but were food restricted (13-25g rat chow/day) throughout experiments except during surgery and recovery. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee in accordance with National Institutes of Health guidelines.

Drugs

Cocaine HCl (National Institute of Drug Abuse) was dissolved in sterile 0.9% saline and administered intravenously (i.v.) at 0.25mg/infusion. For some rats, vehicle (IgG) or a neutralizing antibody against bFGF (anti-bFGF; Millipore, 05-117) was infused into IL-mPFC (0.35µg/0.35µl/side at a rate of 0.15µl/minute) one hour prior to extinction. This neutralizing antibody against bFGF blocks the biological function of bFGF, is highly selective for bFGF with no cross-reactivity to acidic FGF (aFGF or FGF1; Matsuzaki, Yoshitake, Matuo, Sasaki, & Nishikawa, 1989; Tao et al., 1997), and has been used previously (Flores et al., 2000; Hafenbreidel et al., 2015; Mueller et al., 2006).

Self-administration

Apparatus. Sucrose and cocaine self-administration procedures were conducted using 12 standard sound-attenuated operant conditioning chambers (MED Associates) as previously described (Hafenbreidel et al., 2014). Briefly, each chamber (30.5 cm long x 24.1 cm wide x 21.0 cm high) had two retractable levers, two stimulus lights located above each lever, a food hopper placed between the two levers, a house light (25 W), a rod grid floor (23 4.8-mm stainless steel bars spaced 1.3 cm apart), and a speaker/tone generator (65 Hz, 65 dB). Each chamber was located within a sound- and light-attenuating enclosure with a ventilation fan. A syringe pump was located outside of the enclosure and is connected to a single channel swivel (Instech Solomon) by tygon tubing and connector cannula (Plastics One; a metal spring attached to a metal spacer with PE50 tubing inserted down the center) that was connected to the catheter anchored in the rat's back (see *Surgery*).

Lever press training. For overall timeline of methods, see Figure 11. One week after arrival, rats were food restricted for three days prior to a 10-hour sucrose training session on a FR1 schedule of reinforcement. At the beginning of each session, the house light and right stimulus light turned on, and the right lever was extended. Active lever presses resulted in delivery of a 20mg sucrose pellet. When criteria were met (100+ responses), sessions were shortened to 90 min on a FR1 schedule, with the criteria of 50+ responses per session.

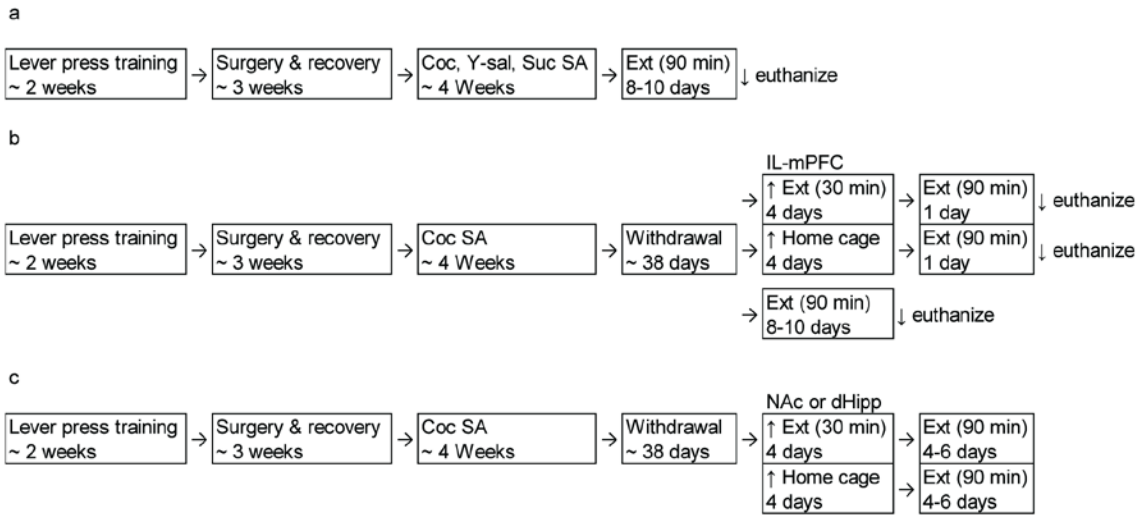

Figure 11: Timeline of methods for Aim one, experiment one (a) and two (b), and Aim two (c).

Surgery. Rats assigned to the yoked-saline or cocaine self-administration conditions were anesthetized with a mixture of ketamine/xylazine (87/13 mg/kg, i.p.) and implanted with chronic intravenous catheters (assembled by Access Technologies) as described previously (Hafenbreidel et al., 2014). Briefly, two incisions were made on the back of the rat for placement of the catheter, and another incision was made over the right jugular vein. A small polyurethane tube was inserted into the jugular vein. The tube was attached to a larger polyurethane tube that was connected to a backmount pedestal (Plastics One), which was implanted behind the shoulder blades. Following catheter implantation, some rats were place in a stereotaxic instrument and implanted with a 26-gauge double barrel guide cannula aimed bilaterally at IL-mPFC (AP, +2.8, ML, ±0.6, DV, -4.4mm relative to bregma), as previously described (Hafenbreidel et al., 2015; Otis, Fitzgerald, & Mueller, 2014). Following surgery, rats received the antibiotic penicillin g procaine (75,000 units/0.35ml, s.c.) and the inflammation and pain reliever carprofen (5mg/0.1ml, s.c.). Rats recovered for a minimal of five days before catheter

patency was verified with 0.3ml of 1% Propofol (i.v.), a short acting anesthetic that causes immediate loss of muscle tone. Rats recovered for a minimum of seven days prior to behavioral testing. Catheter patency was maintained daily with heparinized saline (~0.2 cc of 60 i.u./ml).

Acquisition of cocaine self-administration. Rats in the cocaine self-administration condition were trained to self-administer cocaine daily for 90-min sessions on a FR1 schedule or until the progressive daily cap is achieved (20-35 infusions/session). At the beginning of each session, the house light and right stimulus light turned on, and the right and left lever extended. Following a lever press on the active lever (right), the stimulus light turned off for 20 seconds, a tone (65 dB) sounded for five seconds, and a cocaine infusion was initiated for three seconds. To reduce the risk of overdose, another infusion was not be available until the stimulus light turned on again (time-out period). All lever presses, reinforced or not, were reported as active lever presses during acquisition, extinction, and cocaine-induced reinstatement. Responding on the inactive lever (left) was recorded, but did not have any programmed consequences. For experiment one, a yoked-saline control group was included. These rats were yoked to a cocaine-administering rat throughout acquisition and extinction (if applicable), receiving identical cues, but a saline infusion when the cocaine-administering rat pressed the active lever, and lever presses resulted in no programmed consequences. Rats were trained to self-administer cocaine for 18 sessions when stable drug seeking was achieved. In order to obtain stable drug seeking across rats, some rats received a priming infusion at the start of the session or additional acquisition sessions. Groups were matched on total number of infusions during self-administration, the average

number of infusions over the last three days of self-administration, and if rats receive priming infusions or extra sessions.

To control for motor activity, a separate group of rats continued to lever press for sucrose (FR1), as during initial lever press training, for 90-min daily session or until the progressive daily cap is achieved (50-95 presses/session). At the beginning of each session, the house light and right stimulus light turned on, and the right lever extended. Active lever presses resulted in the delivery of a 20mg sucrose pellet. These rats did not receive catheters or cannula implants, but were trained on the same days as the cocaine and yoked-saline self-administration rats. Groups were matched on the average number of active lever presses.

Experimental manipulations

Experiment one. For overall timeline of methods, see Figure 11a. To determine if extinction alters plasticity following drug self-administration in IL-mPFC, NAc, or dHipp, protein expression was examined following withdrawal or extinction. Specifically, protein expression of ARC, β-catenin (total or phosphorylated form; pβ-catenin), bFGF, Δ-FosB, GluR1, GSK-3β (total or phosphorylated form; pGSK-3β), Kal-7, MAP2, and PSD95 was measured.

Following acquisition, rats were matched and assigned to groups: extinction (coc-ext, yoked-sal-ext, and suc-ext) or no extinction (coc-noext, yoked-sal-noext, and suc-noext). Extinction of sucrose seeking, cocaine seeking, or yoked-saline administration was conducted identical to acquisition, except that no reinforcement was delivered following a lever press. A naïve home-cage control was included that was neither

manipulated nor handled (naïve; experiment 2, Table 1). Rats were given a three-day break before coc-ext, yoked-sal-ext, and suc-ext rats underwent daily 90-min extinction sessions. Coc-noext, yoked-sal-noext, and suc-noext rats remained in their home cages during these sessions. Following the last day of extinction, all groups (except naïve) were tested for extinction retention with the same extinction procedure used throughout the experiment. Immediately after testing, rats were anesthetized with isoflurane gas and their brains were extracted and frozen at -80°C for western blot analysis.

Experiment two. For overall timeline of methods, see Figure 11b. To determine if extinction or bFGF alters plasticity following prolonged withdrawal from cocaine self-administration, protein expression was examined in a number of groups in reward- and learning-related brain regions (IL-mPFC, NAc, and dHipp). The same proteins that were examined in experiment one were examined here.

Following acquisition, rats were matched and assigned to groups: extinction (coc-veh-ext or coc-anti-bFGF-ext) or no extinction (coc-veh-noext or coc-anti-bFGF-noext). Based on preliminary findings (Figure 6), a few controls were excluded, including sucrose self-administration and yoked-saline, but a naïve home cage control was included (Table 4). Rats underwent 36 days of withdrawal before rats were adapted to microinfusion procedures, which included gently restraining rats on the first day (withdrawal day 36), lowering injectors extending 1.0mm past the guide cannula on the second day (withdrawal day 37), and infusing saline at the same rate and volume as during drug manipulation on the third day (withdrawal day 38).

Experiment	n	Treatment	Euthanize day	Tissue collected
1	7	Naïve	Ext retention test	IL, NAc, & dHipp
	8	Suc NE	Ext retention test	
	9	Suc E	Ext retention test	
	12	Y-Sal-NE	Ext retention test	
	11	Y-Sal-E	Ext retention test	
	12	Coc NE(3)	Ext retention test	
	12	Coc E(3)	Ext retention test	
2	13	Naïve	1/2 Ext D5; 1/2 Ext retention test	IL, NAc, & dHipp
	11	Veh NE	Ext D5	
	10	Anti-bFGF NE	Ext D5	
	9	Veh E	Ext D5	
	10	Anti-bFGF E	Ext D5	
	10	Coc NE(38)	Ext retention test	
	8	Coc E(38)	Ext retention test	

Table 4: List of experimental groups and tissue collected for each experiment in Aim one.

On the following day, vehicle (IgG) or anti-bFGF was infused into IL-mPFC one hour before four daily 30-min extinction sessions. Another group of rats also received vehicle or anti-bFGF infusions, but were immediately placed back in their home cages and were not exposed to the operant chambers. Thirty-min sessions were used, as previously described (see Self-administration paradigm section), to better manipulate initial extinction learning. Rats received a two-day break for drug-washout before extinction retention was tested during a 90-min extinction session (extinction day five). During all extinction sessions, lever presses resulted in the same programmed consequences as during self-administration, but no cocaine was administered, in order to model exposure therapy. Immediately following extinction day five, coc-veh-ext, coc-anti-bFGF-ext, coc-veh-noext, coc-anti-bFGF-noext, and half of the naïve rats were anesthetized, and their brains were extracted and frozen at -80˚C.

Additionally, two groups of rats (coc-ext and coc-noext; Table 3) did not receive cannula implants or microinfusions, but did receive the same 38-day withdrawal period

before undergoing extinction (90-min sessions) or remaining in their home cages as described in experiment one. Following the last day of extinction, both groups were tested for extinction retention with the same extinction procedure used throughout the experiment. Immediately after testing, all rats (coc-ext, coc-noext, and half of naïve) were anesthetized with isoflurane gas and their brains were extracted and frozen at -80˚C.

Western blots

To determine the effect of extinction and bFGF on extinction following cocaine self-administration, expression of a number of plasticity-related proteins were measured in reward- and learning-related brain regions: IL-mPFC, NAc, and dHipp. These included ARC, β-catenin (total and phosphorylated), bFGF, Δ-FosB, GluR1, GSK-3β (total and phosphorylated), Kal-7, MAP2, and PSD95. Tissue was collected and processed as previously described (Fortress, Heisler, & Frick, 2015; Hafenbreidel et al., 2014). Briefly, IL-mPFC, NAc, and dHipp was quickly dissected from frozen blocked tissue, and stored at -80˚C until they were re-suspended at 1:25w/v in lysis buffer, homogenized using a probe sonicator (Branson Sonifier 250). Samples were electrophoresed on 18% or 4-15% Tris-HCl gels (Bio-Rad), and transferred to PVDF membranes using a TransBlot Turbo system (Bio-Rad). Membranes were blocked in 5% or 8% (bFGF only) milk for one hour at room temperature, followed by overnight incubation at 4˚C in primary antibodies for ARC (1:100; Santa Cruz), β-Actin (1:5000; Cell Signaling), β-catenin, pβ-catenin (both 1:1000; Cell Signaling), bFGF (1:1000; Millipore), Δ-FosB (1:1000; Santa Cruz), GluR1 (1:1000; Pierce), GSK-3β, pGSK-3β,

(both 1:1000; Cell Signaling), Kal-7 (1:500; Millipore), MAP2 (1:2000; Millipore), and PSD95 (1:1000; Cell Signaling). Next, membranes were incubated in secondary antibodies and developed using West Dura chemiluminescence (Pierce). Protein expression was imaged (ChemiDocMP gel imager, BioRad) and quantified by densitometry using Carestream software. To determine changes in expression, proteins were normalized to β-Actin levels or total protein levels. There were no significant differences between groups in β-Actin protein expression in IL-mPFC, NAc or dHipp (Fs<3.250, ps>0.05). Data is expressed as percent immunoreactivity relative to naïve controls.

Data analysis

Lever presses and infusions during acquisition were analyzed by comparing the average of the last three days of cocaine self-administration between groups using *t*-tests or one-way analysis of variance (ANOVA). For extinction, lever presses were analyzed across days using an repeated-measures ANOVA or across days and between groups using two-way repeated-measures ANOVA. During the extinction retention test, lever presses were analyzed using *t*-tests between groups. For Western blot analysis, mean optical densities were analyzed using a one-way ANOVA. All *post-hoc* tests were conducted, when appropriate, using Fisher's least significant difference (LSD) test.

Aim two: Determine the functional relevance of extinction-induced reduction of bFGF in NAc and dHipp.

Subjects

Same as in Aim one.

Drugs

Same as in Aim one. For some rats, vehicle (0.1% BSA/sterile PBS) or bFGF (R&D Systems, 3339-FB) was infused into IL-mPFC (200ng/0.75µl/side at a rate of 0.25µl/minute) one hour prior to extinction of sucrose seeking. This dose, volume, infusion rate, and time point were based off of previous research (Graham & Richardson, 2011a; Turner et al., 2009; Wagner et al., 1999).

Self-administration

Apparatus. Same as in Aim one.

Lever press training. For overall timeline of methods, see Figure 11c. Training was the same as in Aim one.

Surgery. Rats were anesthetized and implanted with a catheter followed by a 26-gauge double barrel guide cannula aimed bilaterally at NAc (AP, +1.5, ML, ±0.6, DV, -7.0mm relative to bregma) or two single-barrel guide cannula aimed bilaterally at dHipp (AP, -3.5, ML, ±2.6, DV, -3.0mm relative to bregma), as described in Aim one. A separate group of rats did not receive catheters, but were implanted with a 26-gauge double barrel guide cannula aimed bilaterally at IL-mPFC, as described in Aim one.

Post-surgical care, recovery times, and catheter patency and maintenance were conducted the same as in Aim one.

Acquisition of cocaine self-administration. Rats that were implanted with catheters were trained to self-administer cocaine, and groups were subsequently matched as in Aim one. Rats only implanted with cannula were trained to self-administer sucrose, and subsequently matched, as in Aim one.

Experimental manipulations

Experiment three. To determine the role of bFGF in NAc and dHipp during extinction, bFGF was neutralized one hour before four daily 30-min extinction sessions and retention was tested on the following 90-min drug-free extinction session. Following acquisition, rats were matched and assigned to groups (Table 5): extinction (coc-veh-ext, or coc-anti-bFGF-ext) or no extinction (coc-veh-noext or coc-anti-bFGF-noext) for each brain region. Rats underwent a 36-day withdrawal period before being adapted to microinfusion procedures as described in Aim one (injectors extended 1.0mm for NAc or 0.5mm for dHipp past the guide cannula). Similarly to Aim one, vehicle or anti-bFGF was infused one hour before four 30-min extinction sessions or rats were immediately returned to their home cages, which was followed by a two-day break before extinction retention was tested during 90-min extinction sessions (all groups). Extinction was conducted the same as in Aim one.

Experiment four. To determine if overexpression of bFGF in IL-mPFC was sufficient to disrupt new learning, bFGF was infused one hour before four daily 90-min extinction sessions and retention was tested on the following 90-min drug-free extinction

sessions. Short 30-min extinction sessions were not used here because bFGF has a long half-life (overexpressed is still observed in the spinal fluid 24 h after a systemic injection; Wagner et al., 1999), and because the overall rate of extinction was being examined and not initial extinction learning.

Cocaine-induced reinstatement

Following extinction, rats received a priming injection of cocaine (10 mg/kg; i.p.) before undergoing normal extinction procedures to test for cocaine-induced reinstatement.

Locomotion

To determine if anti-bFGF infusion into IL-mPFC, NAc, or dHipp affected general motor activity, rats were infused with vehicle or anti-bFGF one hour prior to being placed in locomotion activity chambers for 20 min.

Group	n	Cannula	Treatment	Ext (30 min)	Ext (90 min)
1	7	NAc	Veh	✓	✓
2	9	NAc	anti-bFGF	✓	✓
3	8	dHipp	Veh	✓	✓
4	9	dHipp	anti-bFGF	✓	✓
5	8	NAc	Veh	X	✓
6	8	NAc	anti-bFGF	X	✓
7	7	dHipp	Veh	X	✓
8	8	dHipp	anti-bFGF	X	✓

Table 5: List of experimental groups for Aim two.

Data analysis

Data was analyzed the same as in Aim one. For locomotor activity, total photobeam breaks between groups were analyzed using a *t*-test. After behavioral procedures, verification of injector tip location was performed on cresyl violet-stained coronal sections, and rats with incorrect placements were removed from analysis.

AIM ONE

RESULTS

Behavioral results

Stimulant drug administration can induce a number of long- immediate early gene lasting plastic changes, including increased bFGF expression in a number of reward-related brain regions. The increase in bFGF may be a neuroprotective response, but prolonged overexpression may induce maladaptive changes that underlie the preservation of drug seeking. However, we previously demonstrated that extinction of drug seeking can reverse the increase in bFGF. In order to determine if extinction or bFGF mediates other drug-induced plasticity changes, we obtained tissue from rats that had undergone cocaine self-administration with or without extinction, in addition to a number of controls, from three brain regions implicated from our preliminary data, including the IL-mPFC, NAc, and dHipp.

Rats first acquired sucrose (Suc), yoked-saline (Y-sal), or cocaine (Coc) self-administration. Active or inactive lever presses and number of infusions (where applicable) were equivalent between Suc, Y-sal, or Coc groups across the average of the last three days of self-administration (Table 3 and 6). Following acquisition, some rats (Coc-ext), Y-sal-ext, Suc-ext) underwent extinction (E), while other rats (Coc-noext, Y-sal-noext, and Suc-noext) remained in their home cages (no extinction; NE). For active lever presses during extinction of sucrose seeking (Figure 5a), ANOVA revealed a significant effect of day ($F_{7,56}$=34.561, p<0.0001), indicating that rats reduced lever presses across days. After the last day of extinction, both groups were tested for extinction retention (Figure 5b). For active lever presses, Suc-ext rats pressed less

than Suc-noext rats (t_{15}=-6.031, p<0.0001), demonstrating good extinction retention for the Suc-ext group. Likewise, ANOVA revealed a significant effect of active lever presses in the Coc-ext group across extinction days ($F_{7,77}$=23.731, p<0.0001), indicating rats learned extinction (Figure 5c). For inactive lever presses (data not shown), ANOVA revealed a significant reduction in lever pressing across days for Coc-ext rats ($F_{7,77}$=9.660, p<0.0001). Comparing active and inactive lever presses across extinction days for Coc-ext rats revealed a significant effect of lever ($F_{1,176}$=154.796, p<0.0001), indicating that Coc-ext rats lever pressed significantly more on the active lever than the inactive lever throughout (data not shown). Active (Figure 5e) and inactive (data not shown) lever presses in the Y-sal-ext group did not differ throughout extinction (active: $F_{7,70}$=1.446, p=0.201; inactive: $F_{7,70}$=0.469, p=0.854). Following the last day of extinction, all rats were tested for extinction retention. Coc-ext rats pressed less than Coc-noext rats on active levers (t_{22}=-3.208, p=0.004), demonstrating good extinction retention (Figure 5d). However, Y-sal-ext rats and Y-sal-noext rats did not differ on lever pressing (t_{21}=-1.161, p=0.259; Figure 5f), as expected. Inactive lever presses (data not shown) during the extinction retention test did not differ between Coc-ext and Coc-noext (t_{22}=-1.981, p=0.060) or Y-sal-ext and Y-sal-noext rats (t_{21}=1.267, p=0.219). Following the extinction retention test, rats were sacrificed and IL-mPFC, NAc, and dHipp tissue was collected for analysis (Figures 6, 14, 15, 17, and 18).

To further determine the role of bFGF in drug-associated plasticity, another group of rats were trained to self-administer cocaine. Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 6).

Experiment	Treatment	n	Active lever	Inactive lever	Infusions
1	Naïve	7	-	-	-
	Suc NE	8	88 ± 4.23	-	-
	Suc E	9	87 ± 5.56	-	-
	Y-Sal-NE	12	6.89 ± 1.93	3.14 ± 0.71	-
	Y-Sal-E	11	5.24 ± 1.72	3.55 ± 1.58	-
	Coc NE(3)	12	37.83 ± 4.08	4.94 ± 3.11	27.56 ± 1.90
	Coc E(3)	12	39.81 ± 4.21	0.56 ± 0.23	28.22 ± 1.45
2	Naïve	13	-	-	-
	Veh NE	11	40.00 ± 6.19	2.79 ± 1.54	27.24 ± 1.57
	Anti-bFGF NE	10	33.93 ± 2.80	1.40 ± 0.75	27.50 ± 1.28
	Veh E	9	36.82 ± 4.33	15.67 ± 14.21	26.58 ± 2.03
	Anti-bFGF E	10	35.55 ± 3.81	2.15 ± 1.68	27.21 ± 1.50
	Coc NE(38)	10	37.83 ± 3.39	0.37 ± 0.20	28.43 ± 1.50
	Coc E(38)	8	42.74 ± 5.28	23.78 ± 23.32	28.48 ± 1.42

Ta**ble 6:** Average number of active and inactive lever presses, or infusions across the last three days of cocaine self-administration, for each experiment. There were no significant differences between groups.

Following acquisition, rats underwent 38 days of withdrawal in their home cages. Next, some rats received vehicle or anti-bFGF (Veh E, Ant-bFGF E) infusions into IL-mPFC one hour before four 30-min extinction sessions. Across the 30-min sessions (days 1-4, active lever presses; Figure 12b), ANOVA revealed a significant effect of day ($F_{3,68}$=17.213, p<0.0001), but no significant effect of treatment ($F_{1,68}$=3.687, p=0.059) or day by treatment interaction ($F_{3,68}$=0.922, p=0.435). During the 90-min extinction retention test (day 5; Figure 12c), anti-bFGF-infused rats lever pressed significantly less than veh-infused rats (t_{17}=-2.433, p=0.026). These results indicate that both groups demonstrate extinction during extinction days 1-4, but that neutralizing bFGF in IL-mPFC significantly reduced lever pressing compared to controls when extinction retention was tested.

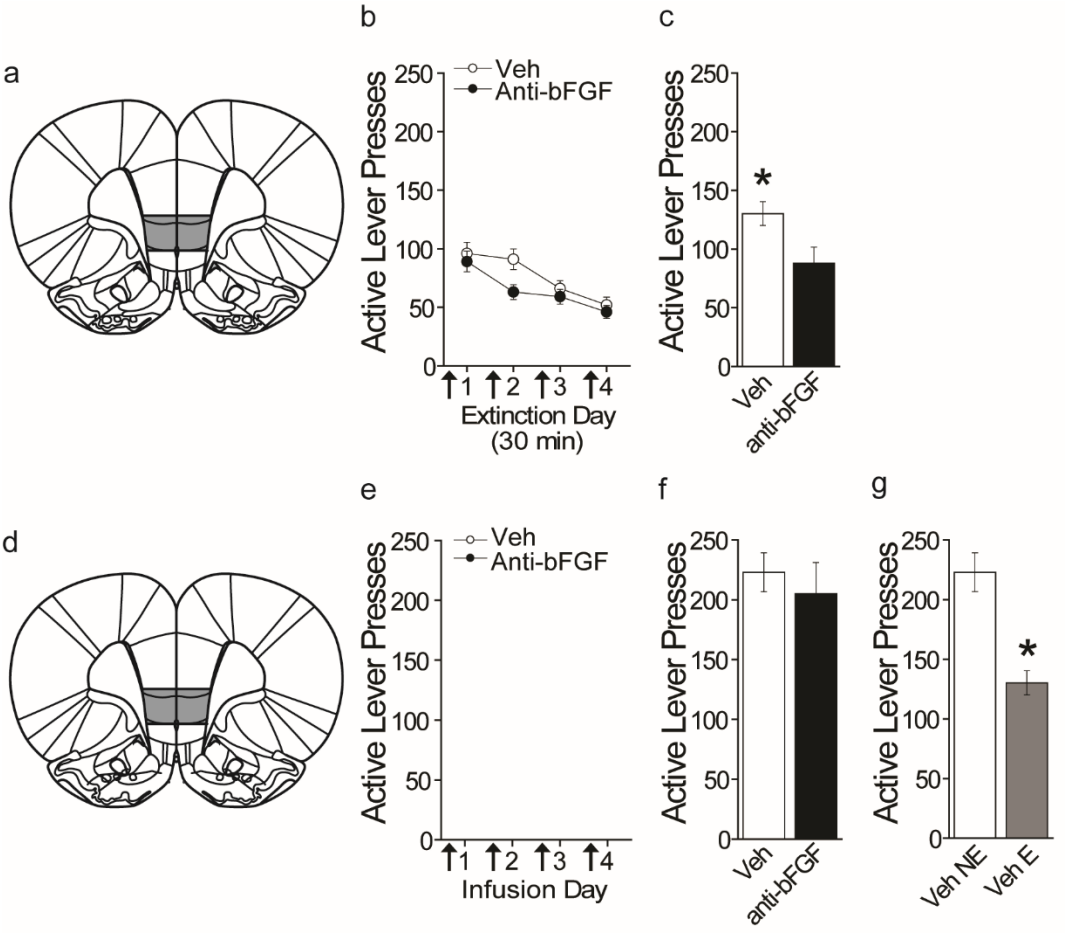

Figure 12: Extinction and extinction retention tests prior to protein analysis in veh- or anti-bFGF infused rats. (a) Representative area for injector tip locations in IL-mPFC; veh: n= 9, anti-bFGF: n=10. (b) Pre-extinction session infusions of anti-bFGF into IL-mPFC (arrows) during the 30-min extinction sessions facilitated extinction retention compared to vehicle infusions (c). (d) Representative area for injector tip locations in IL-mPFC; veh: n= 11, anti-bFGF: n=10. (e) Infusions of either vehicle or anti-bFGF into IL-mPFC for four days (arrows), during which rats were returned immediately to their home cages, did not affect subsequent extinction (f). (g) Veh-infused rats that underwent four 30-min extinction sessions lever pressed less than Veh-infused rats that did not undergo extinction. *p<0.05 Error bars are ±SEM.

For inactive lever presses during extinction days 1-4 (data not shown), ANOVA revealed a significant effect of treatment ($F_{1,68}$=5.582, p=0.021), but no effect of day ($F_{3,68}$=2.631, p=0.057) or day by treatment interaction ($F_{3,68}$=0.370, p=0.775). Comparing active and inactive lever presses across extinction days revealed a

significant effect of lever ($F_{1,136}$=539.379, p<0.0001), indicating that rats pressed significantly more on the active lever than the inactive lever throughout, which demonstrates goal-directed behavior (data not shown). For inactive lever presses during the extinction retention test, anti-bFGF-infused rats lever pressed less on the inactive lever than veh-infused rats (t_{17}=-2.696, p=0.015), but a subsequent ANOVA comparing active and inactive lever presses revealed a significant effect of lever ($F_{1,34}$=109.792, p<0.0001), indicating that rats pressed significantly more on the active lever than the inactive lever (data not shown). Following the extinction retention test, rats were sacrificed and IL-mPFC, NAc, and dHipp tissue was collected for analysis (Figures 6, 14, 15, 17, and 18).

Additionally, to control for infusions of anti-bFGF, another groups of rats received infusions of veh or anti-bFGF into IL-mPFC, but were returned to their home cages without exposure to the operant chamber (Veh NE, Anti-bFGF NE). Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 6). Following acquisition, rats underwent 38 days of withdrawal in their home cages, before being infused with veh or anti-bFGF in IL-mPFC for four days, and being immediately returned to their home cages without exposure to the operant chamber (Figure 12e). Following a two-day wash out period, rats then underwent a 90-min extinction test. During the 90-min extinction retention test (day 5; Figure 12f), there were no significant differences between groups (t_{19}=0.587, p=0.564), indicating that neutralizing bFGF in the IL-mPFC without extinction for four days did not affect subsequent extinction. Inactive lever presses were also not significantly different between groups during the extinction

retention test (t_{19}=1.134, p=0.271). Finally, veh-infused rats that did undergo four 30-min extinction sessions lever pressed significantly less than veh-infused rats that did not undergo the four 30-min extinction sessions (t_{18}=4.573, p<0.0001; Figure 12g), which indicates that four 30-min extinction sessions are sufficient for extinction learning to occur. Following the extinction retention test, rats were sacrificed and IL-mPFC, NAc, and dHipp tissue was collected for analysis (Figures 6, 14, 15, 17, and 18).

Finally, an additional group of rats were not implanted with cannula and did not receive veh or anti-bFGF infusions, but instead underwent 38 days of withdrawal following acquistion before undergoing daily 90-min extinction sessions or remaining in their home cages. These rats were included to control for the limitation that prolonged withdrawal can alter plasticity and potentate drug seeking (e.g., Lu et al., 2004; Pickens et al., 2011), as our preliminary behavioral data included a 38-day withdrawal period, but our bFGF protein analysis data did not. For clarity, rats that underwent extinction without the prolonged withdrawal period are referred to as Coc E(3) and rats that did not undergo extinction or the prolonged withdrawal period are referred to as Coc NE(3); whereas, rats that did undergo the prolonged withdrawal period, with or without extinction, are referred to as Coc E(38) and Coc NE(38), respectively (Table 4). Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 6).

Following acquisition, rats underwent 38 days of withdrawal in their home cages before undergoing daily 90-min extinction sessions (Coc E(38)), or remaining in their home cages (Coc NE(38)). For active lever presses during extinction of cocaine

seeking (Figure 13a), ANOVA revealed a significant reduction in lever pressing across days ($F_{11,77}$=30.878, p<0.0001), indicating that rats learned extinction.

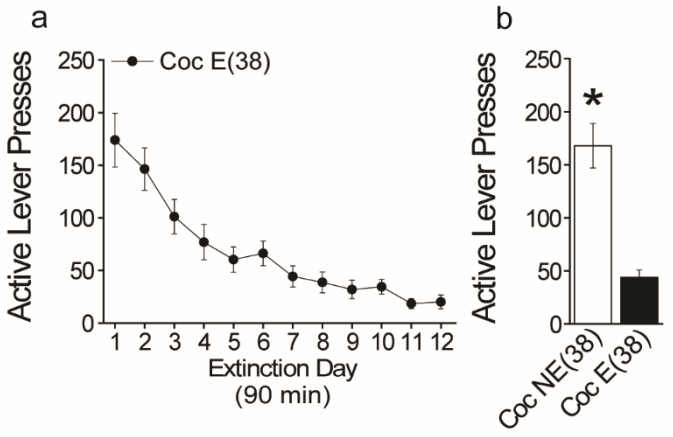

Figure 13: Extinction and extinction retention tests prior to protein analysis in rats that underwent 38 days of withdrawal, but did not receive microinfusions. Active lever presses during extinction of cocaine seeking (a) and during the extinction retention test (b). *p<0.05 Error bars are ±SEM.

For inactive lever presses during extinction (data not shown), ANOVA revealed a significant reduction in lever pressing across days ($F_{11,77}$=3.059, p=0.002). A subsequent ANOVA revealed a significant effect of lever ($F_{1,168}$ =177.692, p<0.0001), which indicates that rats pressed the active lever significantly more than the inactive lever across days (data not shown). During the extinction retention test (Figure 13b), rats that underwent extinction lever pressed significantly less on the active lever than rats that remained in their home cages and did not undergo extinction (t_{16}=-5.279, p<0.0001). For inactive lever presses during the extinction retention test, rats that underwent extinction lever pressed significantly less than rats that remained in their home cages and did not undergo extinction (t_{16}=-3.716, p=0.002; data not shown). However, a subsequent ANOVA revealed a significant effect of lever ($F_{1, 32}$=57.303, p<0.0001), which indicates that rats pressed the active lever significantly more than the inactive lever across days (data not shown). Following the extinction retention test, rats

were sacrificed and IL-mPFC, NAc, and dHipp tissue was collected for analysis (Figures 6, 14, 15, 17, and 18).

Cocaine self-administration induces plastic changes in IL-mPFC, which is partly attenuated by extinction

To determine if extinction alters cocaine-induced plastic changes in IL-mPFC, we used western blots to analyze expression of a number of plasticity-related proteins in rats that underwent cocaine self-administration, followed by a short or long withdrawal period and extinction or no extinction (remained in their home cages). Additionally, if neutralizing bFGF in IL-mPFC had an effect on cocaine-induced plastic changes in rats that underwent four 30-min extinction sessions or remained in their home cages, was examined. We first examined bFGF protein expression to determine the time course of its expression. We next examined a number of plasticity-related proteins, including ARC, GluR1, PSD95, Kal-7, MAP2, β-catenin, and GSK3β, to examine the effect of extinction and bFGF on cocaine-induced plasticity. Figure 14a shows a representative area of tissue collection from IL-mPFC (bregma 3.00 mm; Paxinos & Watson, 2007).

Additionally, efforts were made to examine the protein expression of bFGF's high affinity receptor, FGFR1, in each brain region. However, despite having a functional protocol (e.g., Hafenbreidel et al., 2015), no bands were produced that were viable enough to analyze. Likewise, efforts were made to examine all proteins in each brain region, but due to certain limitations, some proteins were not examined in all brain regions (see Table 7 for summary of all results).

Protein	Brain region	Suc NE	Suc E	Y-Sal-NE	Y-Sal-E	Coc NE(3)	Coc E(3)	Coc NE(38)	Coc E(38)	Veh NE	AB NE	Veh E	AB E
ARC	IL-mPFC	↑	↑	≠	≠	↑	≠	↑	↑	≠	↑	↑	≠
	NAc	≠	≠	≠	≠	↑	≠	↑	↑	↑	≠	≠	↑
	dHipp	≠	≠	≠	≠	≠	≠	↑	≠	↑	↑	↑	↑
bFGF	IL-mPFC	≠	≠	≠	≠	↑	≠	≠	≠	≠	≠	≠	≠
	NAc	≠	↑	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	↑	≠	≠	≠	≠	≠
ΔFosB	IL-mPFC												
	NAc	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp												
GluR1	IL-mPFC	≠	≠	↓	↓	≠	≠	↑	≠	≠	≠	≠	≠
	NAc	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
Kal-7	IL-mPFC	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	NAc	≠	≠	↑	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp												
MAP2	IL-mPFC	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	NAc	≠	≠	≠	≠	≠	≠	≠	↑	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
p-β-cat/T-β-cat	IL-mPFC	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	NAc	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
p-GSK3β/T-GSK3β	IL-mPFC	≠	≠	≠	≠	≠	≠	↓	≠	≠	≠	≠	≠
	NAc	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	↓	↓	≠	≠	≠	≠
PSD95	IL-mPFC	≠	≠	≠	≠	≠	≠	≠	↑	≠	≠	≠	≠
	NAc	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
	dHipp	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠

Table 7: Summary of western blot results.

bFGF protein expression

We previously found that cocaine self-administration increased bFGF protein expression in the IL-mPFC, an effect reversed by extinction (Figure 6), after a relatively short withdrawal period. Next, to determine if self-administration and extinction altered bFGF protein expression in IL-mPFC following a prolonged withdrawal period, we analyzed expression between groups. There were no significant differences in bFGF protein expression between naïve, Coc NE(38), and Coc E(38) rats ($F_{2,23}$=0.788, p=0.466; Figure 14b) indicating that bFGF protein expression is returned to naïve control levels following a prolonged withdrawal period after cocaine self-administration, and extinction does not alter this expression further.

Next, to determine if bFGF protein expression in IL-mPFC is altered following the extinction retention test on day 5, following four days of vehicle or anti-bFGF infusions with or without 30 min extinction sessions (Figure 12), expression was examined between groups. There were no significant differences between groups ($F_{4,47}$=2.138, p=0.091; Figure 14c), but there was a trend for increased expression in groups that underwent cocaine self-administration. These results agree with previous results that bFGF protein expression is not significantly increased following a prolonged withdrawal period. However, they do suggest an increase in bFGF expression when expression is examined during initial extinction learning, even though extinction overall does not appear to affect bFGF protein expression at this time point. Moreover, these results demonstrate that neutralizing bFGF in the IL-mPFC does not persist for three days, as expression is similar between veh- and anti-bFGF infused groups.

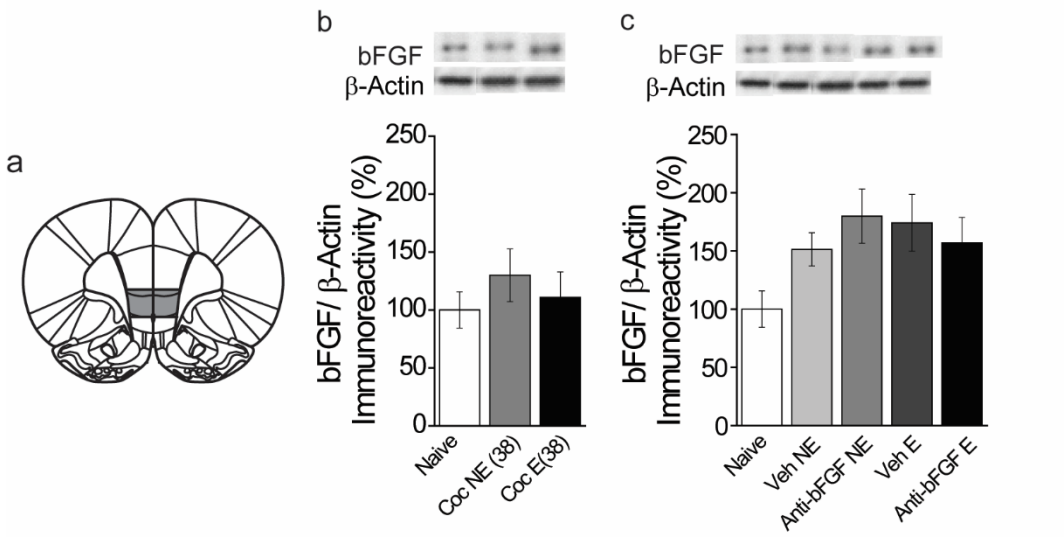

Figure 14: bFGF protein expression is not altered following a prolonged withdrawal period. (a) Representative areas of tissue collection containing IL-mPFC. (b) bFGF protein expression was not altered between naïve (n=13), Coc NE(38) (n=8), or Coc E(38) (n=5) groups. (c) bFGF protein expression was not altered between naïve (n=13), Veh NE (n=11), Anti-bFGF NE (n=10), Veh E (n=9), or Anti-bFGF E (n=9) groups. Representative blots are located above each graph. Error bars are ±SEM.

ARC protein expression

Next, we examined the expression of five proteins associated with cocaine-induced plastic changes, including ARC, GluR1, PSD95, MAP2, and Kal-7 (See Drug-induced Plastic changes section). ARC is an IEG that is increased following stimulant drug administration and increases in its expression are associated with memory retrieval (Alaghband et al., 2014; Caffino et al., 2014; Caffino et al., 2011; Giannotti et al., 2014). To determine if natural reward reinforcement and extinction of reward seeking altered ARC protein expression in IL-mPFC, tissue from naïve, Suc NE, and Suc E groups were compared. ANOVA revealed significant differences between groups ($F_{2,34}$=4.461, p=0.019; Figure 15a). Post hoc tests confirmed that Suc NE rats (p=0.021) and Suc E rats (p=0.023) had higher ARC protein expression than naïve rats,

but Suc NE rats were not significantly different than Suc E rats (p=0.911). These results indicated that ARC protein expression was increased following operant conditioning, but extinction of sucrose seeking does not alter this expression. Next, to determine if cue presentation or i.v. infusions altered ARC protein expression in IL-mPFC, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences, but there was a trend, in ARC protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,39}$=2.986, p=0.062; Figure 15b), indicating that cue presentation and i.v. infusions does not significantly affect ARC protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered ARC protein expression in IL-mPFC, expression was examined between groups (Figure 15c). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,56}$=10.199, p<0.0001). Post hoc tests confirmed that Coc NE(3) rats (p<0.0001), Coc NE(38) rats (p<0.0001), Coc E(38) rats (p=0.003), but not Coc E(3) rats (p=0.415), had higher ARC protein expression than naïve rats. Moreover, Coc NE(3) rats (p=0.012), Coc NE(38) rats (p<0.0001), and Coc E(38) rats (p<0.0001) had higher ARC protein expression than Coc E(3) rats. Finally, there was a trend for decreased ARC expression in Coc E(38) rats compared to Coc NE(38) rats (p=0.068), but it was not significantly different. These results indicate that cocaine self-administration increases ARC protein expression, which is still elevated following a prolonged 38-day withdrawal period. Extinction can reverse this increase following a short 3-day withdrawal period, and is only trending after a longer 38-day withdrawal period.

Finally, to examine ARC protein expression in IL-mPFC after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 15d). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,54}$=3.537, p=0.012). Post hoc tests confirmed that Veh E rats (p=0.026) and Anti-bFGF NE rats (p=0.001) had significantly increased ARC expression compared to naïve rats, and there was a strong trend for Veh NE (p=0.051). Anti-bFGF E rats were not significantly different than naïve rats (p=0.101), and there were no further significant differences between groups (all ps>0.05). These results agree that cocaine self-administration with a 38-day withdrawal period increases ARC protein expression in the IL-mPFC, but suggest that neutralizing bFGF in the IL-mPFC before four 30-min extinction sessions may attenuate ARC protein expression, which matches with lever pressing observed in the behavioral data.

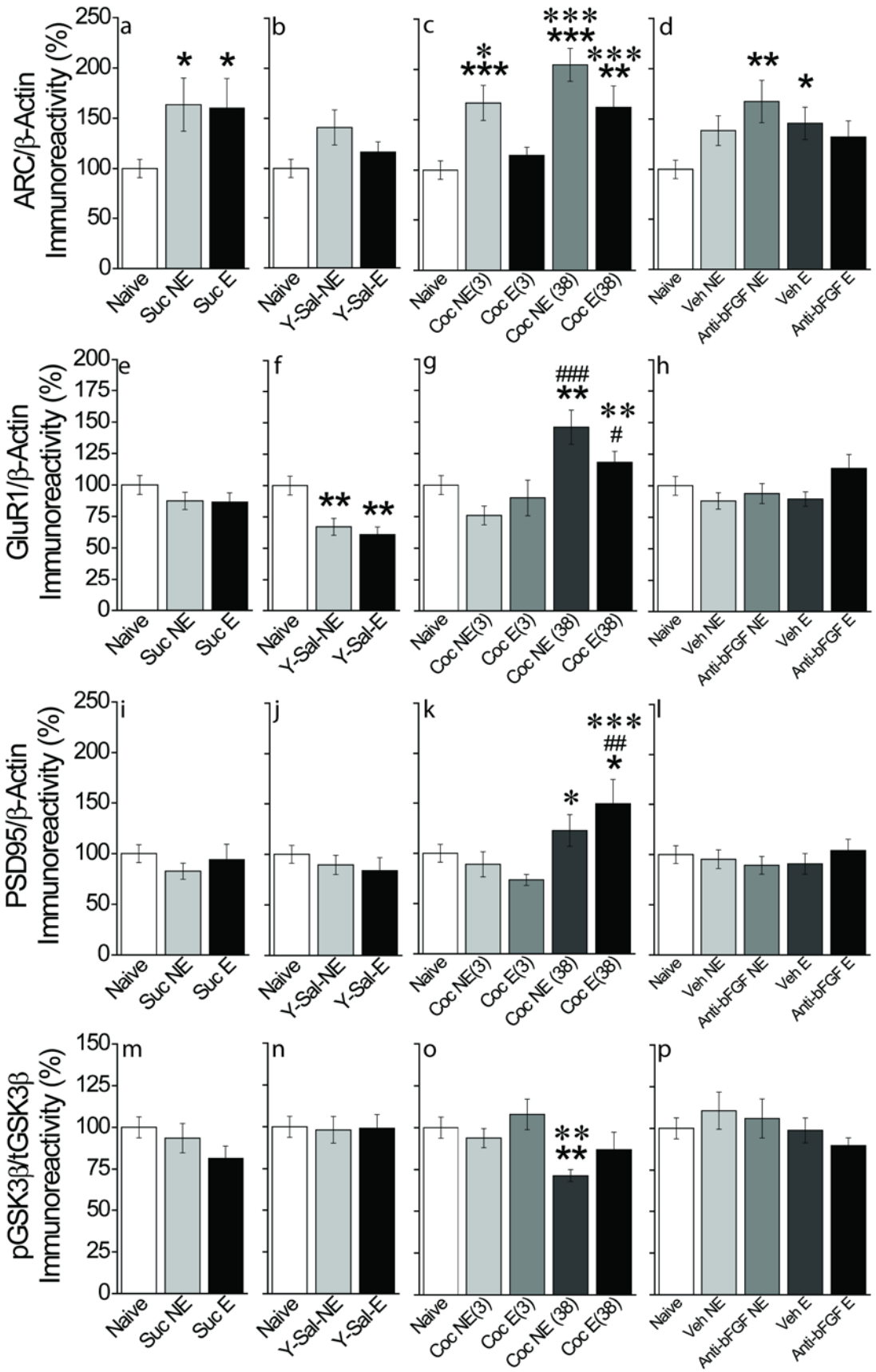
ARC/β-Actin Immunoreactivity (%)
GluR1/β-Actin Immunoreactivity (%)
PSD95/β-Actin Immunoreactivity (%)
pGSK3β/tGSK3β Immunoreactivity (%)
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
Naive
Suc NE
Suc E
Y-Sal-NE
Y-Sal-E
Coc NE(3)
Coc E(3)
Coc NE (38)
Coc E(38)
Veh NE
Anti-bFGF NE
Veh E
Anti-bFGF E

Figure 15: Cocaine self-administration induces plastic changes in IL-mPFC, which are partly attenuated by extinction. (a) Sucrose seeking with or without extinction increased ARC protein expression (naïve, n=20; Suc NE, n=8; Suc E, n=9). (b) ARC protein expression was not altered between naïve (n=20), Y-Sal-NE (n=12), and Y-Sal-E (n=10) rats. (c) ARC protein expression was increased in all groups except Coc E(3) (n=11; Coc NE(3), n=12; Coc NE(38), n=10; Coc E(38), n=8) compared to naïve rats (n=20). (d) ARC protein expression was increased in Anti-bFGF NE (n=10) and Veh E (n=9) rats compared to naïve rats (n=20; Veh NE, n=10; Anti-bFGF E, n=10). (e) GluR1 protein expression was not different between naïve (n=19), Suc NE (n=8), and Suc E (n=8) rats. (f) GluR1 protein expression was decreased in Y-Sal-NE (n=11) and Y-Sal-E (n=10) rats compared to naïve rats (n=19). (g) GluR1 protein expression was increased in rats that underwent cocaine self-administration and long withdrawal (naïve, n=19; Coc NE(3), n=11; Coc E(3), n=12; Coc NE(38), n=10; Coc E(38), n=8). (h) GluR1 protein expression was not different between naïve (n=19), Veh NE (n=11), Anti-bFGF NE (n=10), Veh E (n=8), and Anti-bFGF E (n=10) rats. (i) PSD95 protein expression was not different between naïve (n=19), Suc NE (n=8), and Suc E (n=9) rats. (j) PSD95 protein expression was not different between naïve (n=19), Y-Sal-NE (n=12), and Y-Sal-E (n=10) rats. (k) PSD95 protein expression was increased in rats that underwent cocaine self-administration and long withdrawal (naïve, n=19; Coc NE(3), n=11; Coc E(3), n=11; Coc NE(38), n=10; Coc E(38), n=8). (l) PSD95 protein expression was not different between naïve (n=19), Veh NE (n=11), Anti-bFGF NE (n=9), Veh E (n=8), and Anti-bFGF E (n=10) rats. (m) pGSK3β protein expression was not different between naïve (n=20), Suc NE (n=8), and Suc E (n=8) rats. (n) pGSK3β protein expression was not different between naïve (n=20), Y-Sal-NE (n=12), and Y-Sal-E (n=10) rats. (o) pGSK3β protein expression was decreased only in Coc NE(38) rats (n=9; naïve, n=20; Coc NE(3), n=11; Coc E(3), n=12; Coc E(38), n=8). (p) pGSK3β protein expression was not different between naïve (n=20), Veh NE (n=11), Anti-bFGF NE (n=10), Veh E (n=9), and Anti-bFGF E (n=9) rats. *p<0.05, **p<0.01, ***p<0.001 compared to naïve, #p<0.05, ##p<0.01, ###p<0.001 compared to CNE(3), *p<0.05, **p<0.01, ***p<0.001 compared to CE(3). Error bars are ±SEM.

GluR1 protein expression

GluR1 expression is increased following stimulant drug administration plus a prolonged withdrawal period and increases in its expression are associated with potentiated drug seeking in NAc (Loweth et al., 2014; Wolf & Ferrario, 2010). To determine if natural reward reinforcement and extinction of reward seeking altered GluR1 protein expression in IL-mPFC, tissue from naïve, Suc NE, and Suc E groups were compared. There were no significant differences between groups ($F_{2,32}$=0.958, p=0.394; Figure 15e), which indicates that GluR1 protein expression was not altered following sucrose self-administration with or without extinction. Next, to determine if cue presentation or i.v. infusions altered GluR1 protein expression in IL-mPFC, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. ANOVA revealed significant differences in GluR1 protein expression between groups ($F_{2,37}$=8.699, p=0.001; Figure 15f). Post hoc tests confirmed that Y-Sal-NE rats (p=0.003) and Y-Sal-E rats (p=0.001) had lower GluR1 protein expression than naïve rats, but Y-Sal-NE rats were not different than Y-Sal-E rats (p=0.609). These results indicate that cue presentation and i.v. infusions reduced GluR1 protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered GluR1 protein expression in IL-mPFC, expression was examined between groups (Figure 15g). ANOVA revealed significant differences in GluR1 protein expression between groups ($F_{4,55}$=5.801, p=0.001). Post hoc tests confirmed that Coc NE(38) rats (p=0.002) had significantly increased GluR1 protein expression compared to naïve rats. There was a trend for increased expression in Coc NE(3) rats compared to naïve rats (p=0.085), but Coc E(3)

rats (p=0.451) and Coc E(38) rats (p=0.241) were not significantly different than naïve rats. Moreover, Coc NE(38) rats (p<0.0001) and Coc E(38) rats (p=0.015) had significantly higher GluR1 protein expression compared to Coc NE(3) rats. Coc NE(38) rats (p=0.001), and a trend for Coc E(38) rats (p=0.093), had significantly higher GluR1 protein expression than Coc E(3) rats. Finally, there were no significant differences between Coc NE(3) rats and Coc E(3) rats (p=0.362), or between Coc NE(38) rats and Coc E(38) rats (p=0.112). These results indicate that GluR1 protein expression is increased following prolonged withdrawal from cocaine self-administration, and extinction may attenuate some of these increases.

Finally, to examine GluR1 protein expression in IL-mPFC after an extinction retention test (day 5), following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 15h). ANOVA revealed no significant differences between groups ($F_{4,55}$=1.048, p=0.391. Interestingly, these rats also underwent a 38-day withdrawal period following cocaine self-administration, but do not demonstrate the same significant increase in GluR1 protein expression as Coc NE(38) rats do.

PSD95 protein expression

PSD95 expression is decreased following stimulant drug administration in NAc, and is thought to underlie the reorganization of dendritic spines following drug use (Huang et al., 2009; Yao et al., 2004). To determine if natural reward reinforcement and extinction of reward seeking altered PSD95 protein expression in IL-mPFC, tissue from naïve, Suc NE, and Suc E groups were compared. There were no significant

differences between groups ($F_{2,33}$=0.603, p=0.553; Figure 15i), which indicate that PSD95 protein expression was not altered following sucrose self-administration, with or without extinction. Next, to determine if cue presentation or i.v. infusions altered PSD95 protein expression in IL-mPFC, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences between groups ($F_{2,38}$=0.680, p=0.513; Figure 15j), which indicates cue presentation and i.v. infusions does not significantly affect PSD95 protein expression

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered PSD95 protein expression in IL-mPFC, expression was examined between groups (Figure 15k). ANOVA revealed significant differences in PSD95 protein expression between groups ($F_{4,54}$=4.248, p=0.005). Post hoc tests confirmed that Coc E(38) rats (p=0.010) had significantly increased PSD95 protein expression compared to naïve rats, but Coc NE(3) rats (p=0.520), Coc E(3) rats (p=0.119), and Coc NE(38) rats (p=0.192) were not significantly different than naïve rats. Moreover, Coc E(38) rats had significantly higher PSD95 protein expression than Coc NE(3) rats (p=0.005) and Coc E(3) rats (p<0.0001). Coc NE(38) rats had significantly higher PSD95 protein expression than Coc E(3) rats (p=0.014) and were trending to be higher than Coc NE(3) rats (p=0.087). Finally, there were no significant differences between Coc NE(3) rats and Coc E(3) rats (p=0.410), or between Coc NE(38) rats and Coc E(38) rats (p=0.205). These results indicate that cocaine self-administration increased PSD95 protein expression in IL-mPFC after a prolonged withdrawal period, but not a short withdrawal period. Moreover, extinction does not appear to significantly effect PSD95 protein expression.

Finally, to examine PSD95 protein expression in IL-mPFC after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 15l). ANOVA revealed no significant differences between groups ($F_{4,52}$=0.336, p=0.853). Interestingly, these rats also underwent a 38-day withdrawal period, but do not demonstrate the same significant increase in PSD95 protein expression as the COC E(38) rats do.

Kal-7 and MAP2 protein expression

Kal-7 is increased following cocaine administration, and has a role in cocaine-induced increases in spine density (Kiraly, Ma, et al., 2010; X. Wang et al., 2013). MAP2 is associated with dendritic growth (Gordon-Weeks, 1991; Shafit-Zagardo & Kalcheva, 1998), and is decreased one week following cocaine self-administration, but increased following extinction (Self et al., 2004). However, there were no significant differences between any groups in Kal-7 or MAP2 protein expression in IL-mPFC (all Fs<2.075, *p*s>0.05; data not shown). These results indicate that neither Kal-7 nor MAP2 protein expression, at the selected time points, was altered by cocaine self-administration, extinction of drug seeking, or neutralizing bFGF in IL-mPFC.

β-Catenin and GSK3β protein expression

Lastly, we examined the expression of two proteins, β-catenin and GSK3β, that are known to interact with bFGF signaling to mediate plastic changes, such as proliferation, during development (Boku et al., 2013; Israsena et al., 2004; Shimizu et

al., 2008), and are altered by stimulant drug administration. GSK3β activity is increased (as demonstrated through decreased pGSK3β protein expression) following stimulant drug administration (W. Y. Kim et al., 2013; J. S. Miller et al., 2014; Perrine et al., 2008), which should result in decreased β-catenin activity (as demonstrated through increased pβ-catenin protein expression) though these findings are unclear currently (Dias et al., 2015; Freeman et al., 2001). Therefore, we examined expression of each between groups.

There were no significant differences between any groups in pβ-catenin protein expression (all *F*s<2.353, *p*s>0.05; data not shown), but there were significant differences in pGSK3β expression between groups in IL-mPFC. To determine if natural reward reinforcement and extinction of reward seeking altered pGSK3β protein expression in IL-mPFC, tissue from naïve, Suc NE, and Suc E groups were compared. ANOVA revealed no significant differences between groups ($F_{2,33}$=1.474, p=0.244; Figure 15m). These results indicated that pGSK3β protein expression was not altered by sucrose self-administration, with or without extinction. Next, to determine if cue presentation or i.v. infusions altered pGSK3β protein expression in IL-mPFC, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences between groups ($F_{2,40}$=0.016, p=0.984; Figure 15n), indicating that cue presentation and i.v. infusions does not significantly affect pGSK3β protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered pGSK3β protein expression in IL-mPFC, expression was examined between groups (Figure 15o). ANOVA revealed significant differences in pGSK3β protein expression between groups ($F_{4,55}$=3.042,

p=0.025). Post hoc tests confirmed that Coc NE(38) rats (*p*=0.008), but not Coc NE(3) rats (*p*=0.525), Coc E(3) rats (*p*=0.401), or Coc E(38) rats (*p*=0.226), had lower pGSK3β protein expression than naïve rats. Moreover, Coc NE(38) rats had lower pGSK3β protein expression than Coc E(3) rats (*p*=0.002), were trending to have lower expression than Coc NE(3) rats (*p*=0.058), but were not different than Coc E(38) rats (*p*=0.222). Finally, Coc NE(3) rats were not significantly different than Coc E(3) rats (*p*=0.194). These results indicate that cocaine self-administration decreased pGSK3β protein expression, but only after a prolonged 38-day withdrawal period. Extinction may be able to attenuate this decrease, though not significantly.

Finally, to examine pGSK3β protein expression in IL-mPFC after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 15p). There were no significant differences between groups ($F_{4,54}$=0.713, *p*=0.587). Interestingly, these rats also underwent 38 days of withdrawal following cocaine self-administration, yet they do no demonstrate the same decrease in pGSK3β protein expression as Coc NE(38) rats do.

Cocaine self-administration induces plastic changes in NAc

The NAc has been extensively studied for its role in drug seeking and the long-term plastic changes induced by drug use (see Self-administration paradigm or Drug-induced plastic changes section), however its role in extinction learning is less well characterized. Our preliminary data suggested that bFGF protein expression in the NAc is altered by cocaine self-administration and extinction. Therefore, to determine if

extinction alters cocaine-induced plastic changes in NAc, we used western blotting to analyze protein expression changes following cocaine self-administration with or with extinction. Additionally, if neutralizing bFGF in IL-mPFC had a downstream effect on cocaine-induced plastic changes with or without extinction in the NAc was examined.

Similarly to IL-mPFC, we first examined bFGF protein expression to determine the time course of its expression. Next, we examine ARC, Kal-7, MAP2, GluR1, PSD95, ΔFosB, β-catenin, and GSK3β to examine the effect of extinction and bFGF on cocaine-induced plasticity. Figure 16a shows a representative area of tissue collection from NAc (bregma 2.52 mm; Paxinos & Watson, 2007).

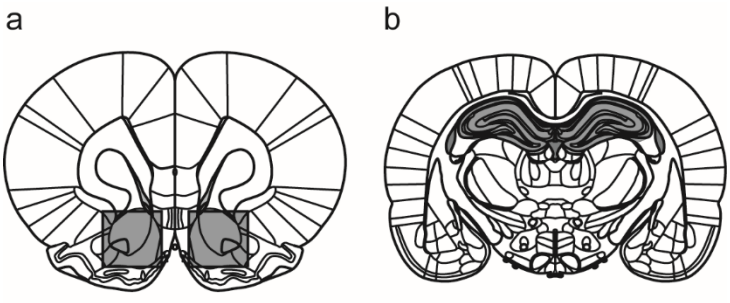

Figure 16: Representative regions of collection containing (a) NAc and (b) dHipp.

bFGF protein expression

Our preliminary data suggested two alternatives for bFGF protein expression in the NAc. When protein expression was examined with western blots, we found that cocaine self-administration increased bFGF protein expression and this was reversed by extinction (Figure 5), which is similar to our findings in IL-mPFC (Figure 6). Alternative, using IHC, we found that extinction increased bFGF immunoreactivity in NAC core and a trend in NAc shell (Figure 8). Due to a number of limitations for each

method, here we attempted to determine how bFGF protein expression was altered in NAc with the inclusion of a number of groups.

To determine if natural reward reinforcement and extinction of reward seeking altered bFGF protein expression in NAc, tissue from naïve, Suc NE, and Suc E groups were compared. ANOVA revealed significant differences between groups ($F_{2,16}$=5.025, p=0.020; Figure 17a). Post hoc tests confirmed that Suc E rats (p=0.006), but not Suc NE rats (p=0.360), had higher bFGF protein expression than naïve rats. There was a trend for Suc E rats to have higher bFGF protein expression than Suc NE rats (p=0.098), but it was not significant. These results indicate that bFGF protein expression was increased following extinction of sucrose seeking in NAc. Next, to determine if cue presentation or i.v. infusions altered bFGF protein expression in NAc, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences in bFGF protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,22}$=2.114, p=145; Figure 17b), which indicate that cue presentation and i.v. infusions does not significantly affect bFGF protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered bFGF protein expression in NAc, expression was examined between groups (Figure 17c). There were no significant differences between groups ($F_{4,29}$=1.044, p=0.402). These results indicate that bFGF protein expression was unchanged in NAc following cocaine self-administration with or without a prolonged withdrawal period, and with or without extinction training. These results do not confirm either preliminary finding.

Next, to determine if bFGF protein expression was altered following the extinction retention test on day 5, following four days of vehicle or anti-bFGF infusions with or without 30 min extinction sessions (Figure 12), expression was examined between groups. There were no significant differences between groups ($F_{4,32}$=1.092, p=0.377; Figure 17d). These results further confirm the previous findings that bFGF protein expression was not changed following prolonged withdrawal or following abridged extinction training. Furthermore, bFGF protein expression in NAc was not affected by neutralizing bFGF in IL-mPFC three days earlier.

ARC protein expression

To determine if natural reward reinforcement and extinction of reward seeking altered ARC protein expression in NAc, tissue from naïve, Suc NE, and Suc E groups were compared. There were no significant differences between groups ($F_{2,27}$=2.486, p=0.102; Figure 17e). These results indicated that ARC protein expression was not altered by sucrose self-administration or extinction of sucrose seeking. Next, to determine if cue presentation or i.v. infusions altered ARC protein expression in NAc, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences, but there was a trend, in ARC protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,37}$=2.564, p=0.091; Figure 17f), indicating that cue presentation and i.v. infusions does not significantly affect ARC protein expression.

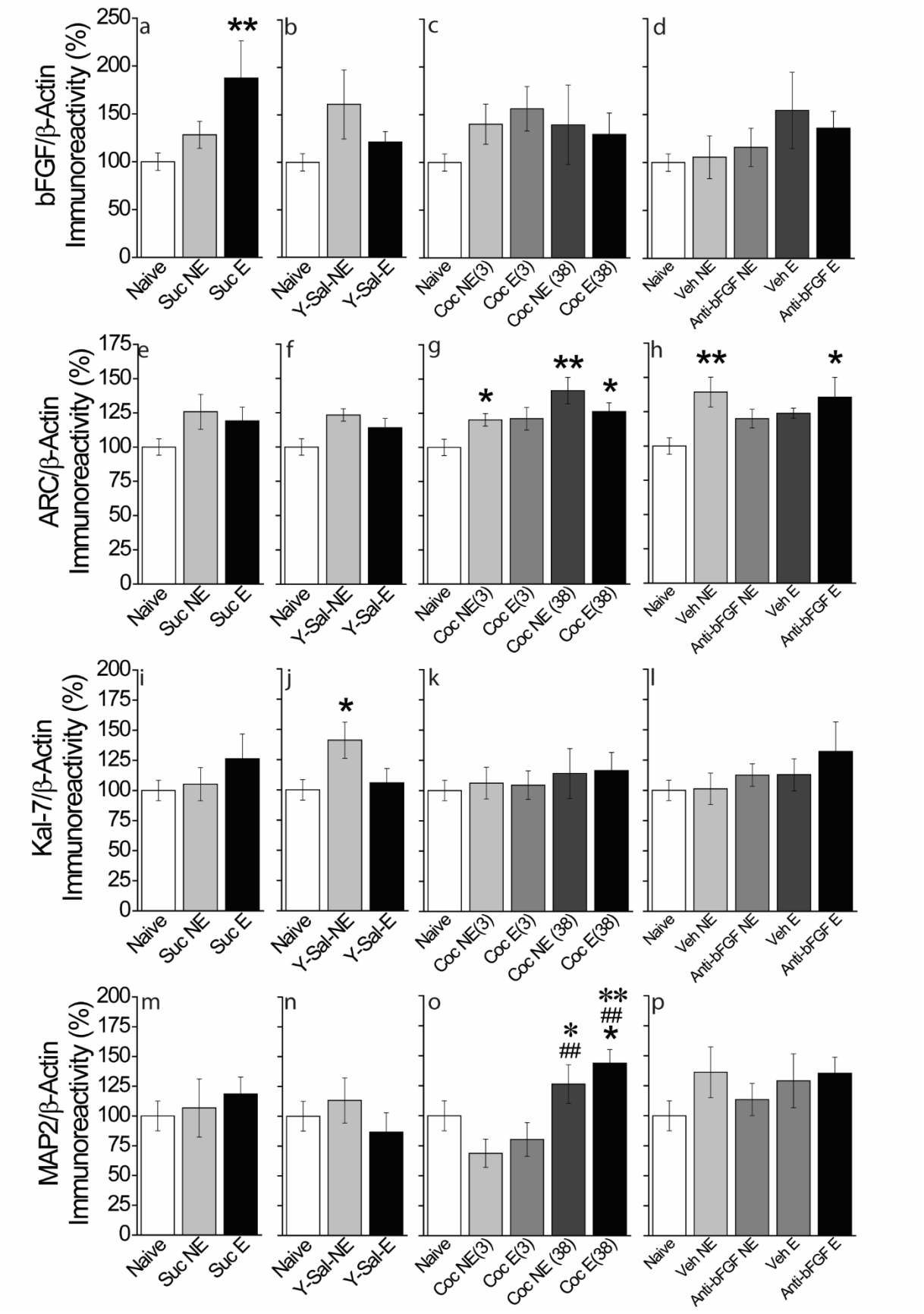
bFGF/β-Actin Immunoreactivity (%)
ARC/β-Actin Immunoreactivity (%)
Kal-7/β-Actin Immunoreactivity (%)
MAP2/β-Actin Immunoreactivity (%)
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
Naive
Suc NE
Suc E
Y-Sal-NE
Y-Sal-E
Coc NE(3)
Coc E(3)
Coc NE (38)
Coc E(38)
Veh NE
Anti-bFGF NE
Veh E
Anti-bFGF E

Figure 17: Cocaine self-administration induces plastic changes in NAc. (a) Extinction of sucrose seeking increased bFGF protein expression (naïve, n=10; Suc NE, n=4; Suc E, n=5). (b) bFGF protein expression was not altered between naïve (n=10), Y-Sal-NE (n=8), and Y-Sal-E (n=7) rats. (c) bFGF protein expression was not altered between naïve (n=10), Coc NE(3) (n=7), Coc E(3) (n=7), Coc NE(38) (n=6), and Coc E(38) (n=4) rats. (d) bFGF protein expression was not different between naïve (n=10), Veh NE (n=7), Anti-bFGF NE (n=7), Veh E (n=6), and Anti-bFGF E (n=7) rats. (e) ARC protein expression was not different between naïve (n=18), Suc NE (n=7), and Suc E (n=5) rats. (f) ARC protein expression was not different between naïve (n=18), Y-Sal-NE (n=11), and Y-Sal-E (n=11) rats. (g) ARC protein expression was increased in all groups, except for Coc E(3) rats (n=10), compared to naïve rats (n=18; Coc NE(3), n=11; Coc NE(38), n=8; Coc E(38), n=8). (h) ARC protein expression was increased in Veh NE (n=11) and Anti-bFGF E (n=7) rats, but not Anti-bFGF NE (n=9) or Veh E (n=8) rats, compared to naïve rats (n=18). (i) Kal-7 protein expression was not different between naïve (n=18), Suc NE (n=6), and Suc E (n=6) rats. (j) Kal-7 protein expression was increased in Y-Sal-NE rats (n=12), but not Y-Sal-E (n=11) rats, compared to naïve rats (n=18). (k) Kal-7 protein expression was not altered between naïve (n=18), Coc NE(3) (n=11), Coc E(3) (n=10), Coc NE(38) (n=8), and Coc E(38) (n=8) rats. (l) Kal-7 protein expression was not different between naïve (n=18), Veh NE (n=11), Anti-bFGF NE (n=9), Veh E (n=8), and Anti-bFGF E (n=7) rats. (m) MAP2 protein expression was not different between naïve (n=17), Suc NE (n=6), and Suc E (n=6) rats. (n) MAP2 protein expression was not different between naïve (n=17), Y-Sal-NE (n=12), and Y-Sal-E (n=11) rats. (o) MAP2 protein expression was increased in rats that underwent cocaine self-administration and long withdrawal (naïve, n=17; Coc NE(3), n=11; Coc E(3), n=10; Coc NE(38), (n=8); Coc E(38), n=8). (p) MAP2 protein expression was not different between naïve (n=17), Veh NE (n=10), Anti-bFGF NE (n=9), Veh E (n=8), and Anti-bFGF E (n=7) rats. *p<0.05, **p<0.01 compared to naïve, #p<0.05, ##p<0.01 compared to CNE(3), *p<0.05, **p<0.01 compared to CE(3). Error bars are ±SEM

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered ARC protein expression in NAc, expression was examined between groups (Figure 17g). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,50}$=3.705, p=0.010). Post hoc tests confirmed that Coc NE(3) rats (p=0.027), Coc NE(38) rats (p=0.001), Coc E(38) rats (p=0.037), and trending in Coc E(3) rats (p=0.081), had increased ARC protein expression compared to naïve rats. However, there were no further differences between groups (all ps>0.05). These results indicate that cocaine self-administration increased ARC protein expression after a short or prolonged withdrawal period, but extinction does not appear to have an affect on ARC protein expression in NAc.

Finally, to examine ARC protein expression in NAc after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 17h). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,48}$=3.357, p=0.017). Post hoc tests confirmed that Veh NE rats (p=0.002), Anti-bFGF E rats (p=0.014), and a trend for Veh E rats (p=0.096), had significantly increased ARC protein expression compared to naïve rats, but no change in expression for Anti-bFGF NE rats (p=0.159). There were no further significant differences between groups (all ps>0.05). These results partly agree that cocaine self-administration followed by a 38-day withdrawal period increases ARC protein expression in the NAc. However, veh-infused rats that underwent four 30-min extinction sessions or anti-bFGF-infused rats that did not undergo four 30 min extinction sessions had similar ARC expression that was not significantly different from naïve rats.

Kal-7 protein expression

To determine if natural reward reinforcement and extinction of reward seeking altered Kal-7 protein expression in NAc, tissue from naïve, Suc NE, and Suc E groups were compared. There were no significant differences between groups ($F_{2,26}$=1.061, p=0.361; Figure 17i). These results indicated that Kal-7 protein expression was not altered by sucrose self-administration or extinction of sucrose seeking. Next, to determine if cue presentation or i.v. infusions altered Kal-7 protein expression in NAc, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. ANOVA revealed significant differences in KAL-7 protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,36}$=3.758, p=0.033; Figure 17j). Post hoc test confirm that Y-Sal-NE rats had higher Kal-7 protein expression than naïve rats (p=0.012) and were trending to have higher Kal-7 protein expression than Y-Sal-E rats (p=0.054). Y-Sal-E rats were not different than naïve rats (p=0.724). These results indicate that Kal-7 was increased following yoked-saline administration.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered KAL-7 protein expression in NAc, expression was examined between groups (Figure 17k). There were no significant differences between groups ($F_{4,49}$=0.282, p=0.888). Moreover, to examine KAL-7 protein expression in NAc after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 17l). There were no significant differences between groups ($F_{4,46}$=0.900, p=0.472). In summary, Kal-7 protein expression was not altered

following cocaine self-administration with a short or long withdrawal point, following extinction, or after bFGF is neutralized in IL-mPFC at the selected time points.

MAP2 protein expression

To determine if natural reward reinforcement and extinction of reward seeking altered MAP2 protein expression in NAc, tissue from naïve, Suc NE, and Suc E groups were compared. There were no significant differences between groups ($F_{2,26}$=0.301, p=0.743; Figure 17m). These results indicated that MAP2 protein expression was not altered by sucrose self-administration or extinction of sucrose seeking. Next, to determine if cue presentation or i.v. infusions altered MAP2 protein expression in NAc, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences in MAP2 protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,37}$=0.630, p=0.538; Figure 17n), which indicates that cue presentation and i.v. infusions does not significantly affect MAP2 protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered MAP2 protein expression in NAc, expression was examined between groups (Figure 17o). ANOVA revealed significant differences in MAP2 protein expression between groups ($F_{4,49}$=4.448, p=0.004). Post hoc tests confirmed that Coc E(38) rats (p=0.027), trending in Coc NE(3) rats (p=0.077), but not Coc E(3) rats (p=0.283) or Coc NE(38) rats (p=0.167), had increased MAP2 protein expression compared to naïve rats. Furthermore, Coc NE(38) rats (p=0.007; p=0.034) and Coc E(38) rats (p=0.001; p=0.005) had significantly higher MAP2 protein expression than Coc NE(3) rats and Coc E(3) rats, respectively. Finally, Coc NE(3) rats

were not different than Coc E(3) rats (p=0.545), and Coc NE(38) rats were not different than Coc E(38) rats (p=0.453), Overall, these results demonstrate that MAP2 protein expression was increased following cocaine self-administration with a prolonged withdrawal, but not a short withdrawal, period in NAc. Additionally, extinction does not affect MAP2 protein expression in NAc at the selected time points.

Finally, to examine MAP2 protein expression in NAc after an extinction retention test (day 5) following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 17p). There were no differences between groups ($F_{4,46}$=1.060, p=0.387). Interestingly, the rats in these groups also underwent a 38-day withdrawal period, but do not demonstrate the same significant increase in MAP2 protein expression as the COC E(38) rats do.

GluR1, PSD95, and ΔFosB protein expression

ΔFosB expression is increased following administration of most commonly abused substances in a number of brain regions (e.g., Atkins et al., 1999; Y. Kim et al., 2009; Larson et al., 2010; Nye et al., 1995; Nye & Nestler, 1996; Perrotti et al., 2008; Pich et al., 1997), and is thought to mediate the motivation to drug seek (Colby et al., 2003). However, there were no significant differences between any groups in ΔFosB, as well as GluR1 or PSD95, protein expression in NAc (all Fs<2.015, ps>0.05; data not shown). These results indicate that GluR1, PSD95, and ΔFosB protein expression in NAc was not altered at the selected time points by cocaine self-administration with a short or long withdrawal period, extinction of drug seeking, or neutralizing bFGF in IL-mPFC.

β-Catenin and GSK3β protein expression

There were no significant differences between any groups in pβ-catenin or pGSK3β protein expression in NAc (all *F*s<2.398, *p*s>0.05; data not shown). These results indicate that pβ-catenin and pGSK3β protein expression in the NAc was not altered at any of the selected time points by cocaine self-administration with a short or long withdrawal period, extinction of drug seeking, or neutralizing bFGF in IL-mPFC.

Cocaine self-administration induces plastic changes in dHipp

Less research is conducted on the role of the dHipp in drug addiction; however, substantial research is conducted on its role in learning and memory (e.g., Fanselow & Dong, 2010; Pergola & Suchan, 2013). Our preliminary data suggested that extinction of cocaine seeking could reduce bFGF protein expression in the dHipp. Therefore, we targeted this region to determine if cocaine self-administration induced plastic changes, and if extinction could mediate these changes. To determine these changes, we analyzed protein expression changes following cocaine self-administration after a short or prolonged withdrawal period, with or with extinction. Additionally, if neutralizing bFGF in IL-mPFC had an indirect effect on cocaine-induced plastic changes with or without extinction in the dHipp was examined. Similarly, to IL-mPFC and NAc, we first examined bFGF protein expression to determine the time course of its expression. Next, we examine ARC, GluR1, PSD95, MAP2, β-catenin, and GSK3β to examine the effect of extinction and bFGF on cocaine-induced plasticity. Figure 16b shows a representative area of tissue collection from dHipp (bregma -3.24 mm; Paxinos & Watson, 2007).

bFGF protein expression

Our preliminary data suggested that extinction of drug seeking following cocaine self-administration reduced bFGF protein expression (Figure 9). Here, we examine bFGF protein expression in dHipp with the addition of a number of treatment groups. To determine if natural reward reinforcement and extinction of reward seeking altered bFGF protein expression in dHipp, tissue from naïve, Suc NE, and Suc E groups were compared. There were no differences between groups ($F_{2,24}$=0.18, p=0.864; Figure 18a), which suggests that bFGF protein expression was not altered following sucrose self-administration or extinction of sucrose seeking in dHipp. Next, to determine if cue presentation or i.v. infusions altered bFGF protein expression in dHipp, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no significant differences in bFGF protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,27}$=0.229, p=796; Figure 18b), which indicates that cue presentation and i.v. infusions does not significantly affect bFGF protein expression in dHipp.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered bFGF protein expression in dHipp, expression was examined between groups (Figure 18c). ANOVA revealed significant differences between groups ($F_{4,42}$=1.044, p=0.402). Post hoc tests confirmed that Coc NE(38) rats (p=0.027) had increased bFGF protein expression compared to naïve rats, but Coc NE(3) rats (p=0.218), Coc E(3) rats (p=0.193), and Coc E(38) rats (p=0.424) were not different than naïve rats. Additionally, Coc NE(38) rats had significantly higher bFGF protein expression than Coc NE(3) rats (p=0.026) and Coc E(3) rats (p=0.003), but were not different than Coc E(38) rats (p=0.245). These results

indicate that bFGF protein expression was increased in dHipp following cocaine self-administration with a prolonged withdrawal, but not short withdrawal, period and extinction may attenuate this increase.

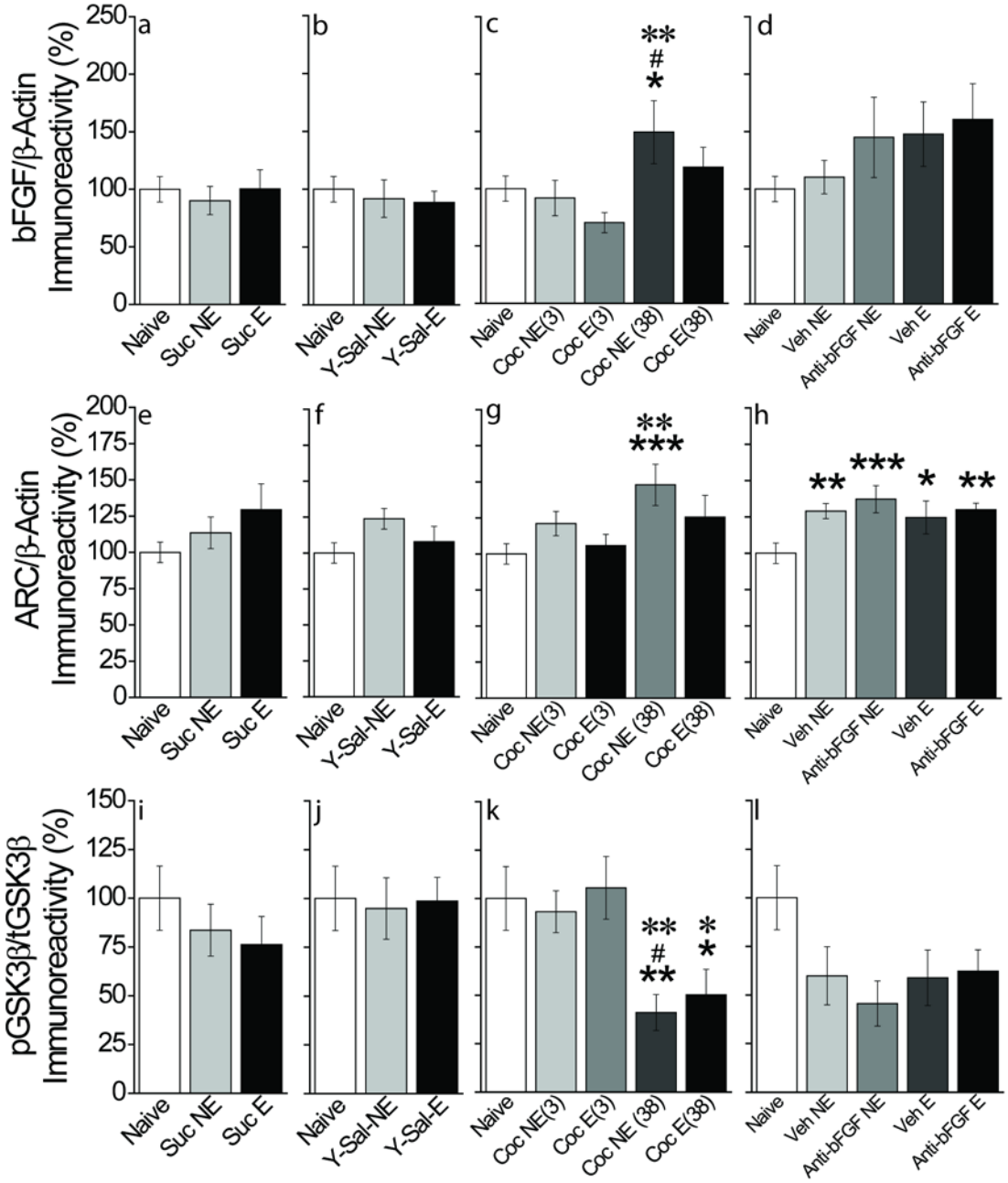

Figure 18: Cocaine self-administration induces plastic changes in dHipp. (a) bFGF protein expression was not different between naïve (n=15), Suc NE (n=6), and Suc E (n=6) rats. (b) bFGF protein expression was not altered between naïve (n=15), Y-Sal-NE (n=8), and Y-Sal-E (n=7) rats. (c) bFGF

protein expression was increased in Coc NE(38) (n=9), but not Coc NE(3) (n=8), Coc E(3) (n=8), or Coc E(38) (n=7) rats, compared to naïve rats (n=15). (d) bFGF protein expression was not different between naïve (n=15), Veh NE (n=11), Anti-bFGF NE (n=9), Veh E (n=9), and Anti-bFGF E (n=8) rats. (e) ARC protein expression was not different between naïve (n=19), Suc NE (n=8), and Suc E (n=9) rats. (f) ARC protein expression was not different between naïve (n=19), Y-Sal-NE (n=11), and Y-Sal-E (n=11) rats. (g) ARC protein expression was only increased in Coc NE(38) rats (n=10; Coc NE(3), n=12; Coc E(3), n=11; Coc E(38), n=8; naïve, n=19). (h) ARC protein expression was increased in all groups (Veh NE, n=11; Anti-bFGF NE, n=10; Veh E, n=9; and Anti-bFGF E, n=10) compared to naïve rats (n=19). (i) pGSK3β protein expression was not different between naïve (n=18), Suc NE (n=6), and Suc E (n=5) rats. (j) pGSK3β protein expression was not different between naïve (n=18), Y-Sal-NE (n=11), and Y-Sal-E (n=10) rats. (k) pGSK3β protein expression was decreased in rats that underwent cocaine self-administration and prolonged withdrawal (naïve, n=18; Coc NE(3), n=10; Coc E(3), n=10; Coc NE(38), n=10; Coc E(38), n=8). (l) pGSK3β protein expression was not different between naïve (n=18), Veh NE (n=11), Anti-bFGF NE (n=8), Veh E (n=9), and Anti-bFGF E (n=10) rats. **p*<0.05, ***p*<0.01 compared to naïve, #*p*<0.05, ##*p*<0.01 compared to CNE(3), **p*<0.05, ***p*<0.01 compared to CE(3). Error bars are ±SEM.

Next, to determine if bFGF protein expression was altered following the extinction retention test on day 5, following four days of vehicle or anti-bFGF infusions with or without 30 min extinction sessions (Figure 12), expression was examined between groups. There were no significant differences between groups ($F_{4,48}$=1.605, p=0.188; Figure 18d). Interestingly, these rats also underwent 38 days of withdrawal following cocaine self-administration, but do not demonstrate significantly increased bFGF protein expression as Coc NE(38) rats do. Furthermore, these results demonstrate that bFGF protein expression in dHipp was not affected by neutralizing bFGF in IL-mPFC three days earlier.

ARC protein expression

To determine if natural reward reinforcement and extinction of reward seeking altered ARC protein expression in dHipp, tissue from naïve, Suc NE, and Suc E groups were compared. There were no differences between groups ($F_{2,33}$=1.908, p=0.165; Figure 18e). These results indicated that ARC protein expression was not altered following sucrose self-administration or extinction of sucrose seeking. Next, to determine if cue presentation or i.v. infusions altered ARC protein expression in dHipp, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no differences in ARC protein expression between naïve, Y-Sal-NE, and Y-Sal-E rats ($F_{2,38}$=2.115, p=0.135; Figure 18f), which indicates that cue presentation and i.v. infusions does not affect ARC protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered ARC protein expression in dHipp, expression was examined between groups (Figure 18g). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,55}$=3.658, p=0.010). Post hoc tests confirmed that Coc NE(38) rats (p=0.001) had significantly higher ARC protein expression compared to naïve rats. There was a trend for increased ARC protein expression in Coc NE(3) rats (p=0.099) and Coc E(38) rats (p=0.083) compared to naïve rats, but Coc E(3) rats (p=0.665) were not different. Moreover, Coc NE(38) rats had higher ARC protein expression than Coc E(3) rats (p=0.007), and were trending to be higher than Coc NE(3) rats (p=0.075). Finally, there were no differences in ARC protein expression between Coc E(3) rats and Coc NE(3) rats (p=0.282), and between Coc E(38) and Coc NE(38) rats (p=0.176). These results indicate that cocaine self-

administration with a long withdrawal period, but not short, increased ARC protein expression in dHipp, and extinction may attenuate this increase.

Finally, to examine ARC protein expression in dHipp after an extinction retention test (day 5), following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 18h). ANOVA revealed significant differences in ARC protein expression between groups ($F_{4,54}$=4.324, p=0.004). Post hoc tests confirmed that Veh NE rats (p=0.007), Anti-bFGF NE rats (p=0.001), Veh E rats (p=0.028), and Anti-bFGF E rats (p=0.006) had significantly increased ARC expression compared to naïve rats. However, there were no further significant differences between groups (ps>0.05). These results agree that cocaine self-administration with a 38-day withdrawal period increased ARC protein expression in dHipp, but here there was no effect of extinction on expression.

GluR1, PSD95, and MAP2 protein expression

There were no significant differences between any groups in GluR1, PSD95, or MAP2 protein expression in dHipp (all Fs<2.061, ps>0.05; data not shown). These results indicate that GluR1, PSD95, and MAP2 protein expression in dHipp was not altered at the selected time points by cocaine self-administration following a short or long withdrawal period, extinction of drug seeking, or neutralizing bFGF in IL-mPFC.

β-Catenin and GSK3β protein expression

There were no significant differences between any groups in pβ-catenin expression (all Fs<1.568, ps>0.05; data not shown); however, there were differences in

pGSK3β in dHipp. To determine if natural reward reinforcement and extinction of reward seeking altered pGSK3β protein expression in dHipp, tissue from naïve, Suc NE, and Suc E groups were compared. There were no differences between groups ($F_{2,26}$=0.395, p=0.678; Figure 18i). These results indicated that pGSK3β protein expression was not altered by sucrose self-administration or extinction of sucrose seeking. Next, to determine if cue presentation or i.v. infusions altered pGSK3β protein expression in dHipp, tissue from naïve, Y-Sal-NE, and Y-Sal-E groups were compared. There were no differences between groups ($F_{2,36}$=0.026, p=0.974; Figure 18j), which indicates that cue presentation and i.v. infusions did not significantly affect pGSK3β protein expression.

Subsequently, to determine if cocaine self-administration with a short or long withdrawal period or extinction of drug seeking altered pGSK3β protein expression in dHipp, expression was examined between groups (Figure 18k). ANOVA revealed significant differences in pGSK3β protein expression between groups ($F_{4,51}$=3.552, p=0.012). Post hoc tests confirmed that Coc NE(38) rats (p=0.005) and Coc E(38) rats (p=0.027), but not Coc NE(3) rats (p=0.733) and Coc E(3) rats (p=0.787), had lower pGSK3β protein expression than naïve rats. Moreover, Coc NE(38) rats had lower pGSK3β protein expression than Coc NE(3) rats (p=0.028) and Coc E(3) rats (p=0.007), and CE(38) rats had significantly lower pGSK3β protein expression than Coc E(3) rats (p=0.027). Finally, Coc NE(3) rats were not significantly different than Coc E(3) rats (p=0.590), and Coc NE(38) rats were not different than Coc E(38) rats (p=0.709). These results indicate that cocaine self-administration decreased pGSK3β protein

expression, but only after a prolonged 38-day withdrawal period, and extinction does not appear to effect pGSK3β protein expression at this time point in dHipp.

Finally, to examine pGSK3β protein expression in dHipp after an extinction retention test (day 5), following four days of veh or anti-bFGF infusions with or without 30-min extinction sessions, expression was compared between groups (Figure 18l). There were no significant differences between groups, though there was a trend ($F_{4,51}$=2.150, p=0.088). Interestingly, these rats also underwent 38 days of withdrawal following cocaine self-administration, yet they do no demonstrate the same decrease in pGSK3β protein expression as Coc NE(38) rats and Coc E(38) rats do.

DISCUSSION

The current experiments aimed to determine if extinction could reverse cocaine-induced plastic changes, and if neutralizing bFGF in IL-mPFC before four extinction sessions could facilitate extinction-induced plastic changes. We observed a number of plastic changes following cocaine self-administration, predominantly following a prolonged withdrawal period, in IL-mPFC, NAc, and dHipp (Table 7). In the IL-mPFC, extinction reversed cocaine-induced increases in bFGF and ARC protein expression, but only after the short withdrawal period. However, in the other proteins of interest, extinction only attenuated some changes. Moreover, specifically neutralizing bFGF in the IL-mPFC before four 30-min extinction sessions did not significantly alter protein expression in any brain region. Overall, these results give insights into proteins to target that could enhance extinction and ultimately reduce relapse rates.

Overall, cocaine self-administration resulted in increased expression of ARC, bFGF, and GluR1 and decreased expression of GSK3β in IL-mPFC; increased expression of ARC in the NAc; and increased expression of ARC and bFGF, and decreased expression of GSK3β in dHipp, compared to naïve controls. Cocaine self-administration, however, had no effect on expression of ΔFosB, Kal-7, and pβ-catenin, in any brain region examined at the selected time points. Alternatively, rats that underwent extinction were not different than naïve in their expression of ARC (after short withdrawal period), bFGF, GluR1, and GSK3β in IL-mPFC; ARC (after short withdrawal period) in NAc; and ARC and bFGF in dHipp. Moreover, PSD95 (IL-mPFC) and MAP2 (NAc) were increased in Coc E(38) rats compared to naïve controls. However, with the exception of ARC and bFGF in IL-mPFC after the short withdrawal

period, Coc NE(3) rats were not significantly different than Coc E(3) rats and Coc NE(38) rats were not significantly different than Coc E(38) rats in any protein of interest, but there were trends. Therefore, extinction does result in changes in protein expression, but these changes may be subtle at the selected time points or reduced after prolonged withdrawal.

Interestingly, in a region-specific manner, cocaine self-administration induced increases in GluR1, PSD95, pGSK3β, MAP2, and bFGF that were observed following a prolonged withdrawal period, but were not observed in rats that were euthanized after extinction day 5 (extinction retention test) in rats that previously received veh- or anti-bFGF infusions, with or without 30 min extinction sessions, for four days. ARC expression, however, was increased in nearly all groups that underwent prolonged withdrawal in all three brain regions. Veh E, Anti-bFGF E, Veh NE, Anti-bFGF NE rats underwent equal acquisition and withdrawal days as the animals that underwent extinction to criteria (Coc E(38)) or remained in their home cages (Coc NE(38)), and therefore they would have been expected to have similar protein expression.

These rats differ from the "full" extinction groups in a few ways. They were implanted with cannula, underwent four days of microinfusions, did not extinguish to criteria, and they were euthanized after 45-days of withdrawal compared to 51-days of withdrawal (Coc NE(38) and Coc E(38) rats). Coc NE(38), Veh NE, and Anti-bFGF NE rats all only underwent one extinction day (extinction retention test), but similar changes between groups were not observed. Therefore, the number of days and amount of time undergoing extinction is unlikely to explain the differences in expression.

The number of withdrawal days may be able to explain the differences, however, the opposite results would be expected (e.g., changes at the early time point). Others have reported that the number of days of stimulant drug treatment, route of administration, and number of withdrawal days can result in differences in spine density and morphology (Dobi et al., 2011; Dumitriu et al., 2012; K. W. Lee et al., 2006), and protein expression such as GluR1, β-catenin, and MAP2 (Dias et al., 2015; Freeman et al., 2010; Self et al., 2004; Wolf, 2010; Wolf & Ferrario, 2010; Yao et al., 2004). Moreover, one experiment even found an inverted "u" shaped curved for β-catenin expression in NAC when expression was examined at 1, 10, or 100 days of withdrawal following 10 continuous days of cocaine self-administration, with expression peaking at 10 days (Freeman et al., 2010). However, in most experiments, protein expression is compared after 24 hours of withdrawal to 14 days. We examined protein expression after 12, 45, or 51 days of withdrawal (from the last day of acquisition), and might have missed changes in protein expression only observed during early withdrawal periods.

Another limitation to our design is that all groups underwent an extinction retention test prior to being euthanized. In groups that did not undergo extinction, one 90-min extinction session might have been sufficient to induce plastic changes in certain brain regions. Alternatively, being placed in the operant chamber prior to being euthanized would reactive the original drug-cue memory and therefore could have induced changes that way. For example, ARC protein expression was increased following retrieval of a cocaine conditioned place preference memory in IL-mPFC and PL-mPFC (Alaghband et al., 2014) and following retrieval of conditioned fear memory in the lateral amygdala (Maddox & Schafe, 2011).

The retrieval of the original drug-cue memory may explain the increase in ARC protein expression in all three brain regions observed in some groups following an extinction retention test. In the IL-mPFC and NAc, ARC protein expression was increased in Coc NE(3), Coc NE(38), and Coc E(38) rats, but not in Coc E(3) rats, compared to naïve. In dHipp, ARC protein expression was only increased in Coc NE(38) rats compared to naïve. These results might be demonstrating a difference in memory retrieval, which differs depending on the withdrawal length. In other words, Coc NE(3) rats retrieved the original drug cue memory resulting in higher ARC protein expression, but Coc E(3) rats retrieved the extinction memory which did not result in higher ARC protein expression. However, after a prolonged withdrawal period, which also corresponded with increased drug seeking compared to a short withdrawal period (see Figure 23), ARC protein expression was increased with or without extinction.

Further research needs to be conducted to confirm these findings, but the results are interesting when considering how protein expression corresponds with differences in active lever pressing during extinction day one and the retention test. To my knowledge, little work has been conducted on the mechanisms underlying retrieval of an extinction memory. In comparison, extinction and reconsolidation share similar, but distinct, underlying mechanisms (Suzuki et al., 2004; Tronson & Taylor, 2007), with some research suggesting they compete at the molecular level (Nader, 2003). Perhaps extinction and retrieval of the original drug-cue memory also compete at the molecular level. This competition could explain why differences in IL-mPFC and NAc are observed in ARC protein expression in Veh NE, Anti-bFGF NE, Veh E, and Anti-bFGF E rats that does not correspond with length of extinction undergone by the rats.

Interestingly, ARC protein expression was increased in all four groups in dHipp, which is important for memory retrieval in a number of paradigms (S. A. Thomas, 2015).

To control for withdrawal length and memory reactivation, future experiments should include a group of rats that are euthanized shortly following the last day of acquisition (e.g., 30 min or 24 hours). This time point would act as a baseline for cocaine-induced changes, and allow comparisons to be made at any addition time points after a withdrawal point (e.g., if expression increases or decreases relative to immediately at the cessation of drug use). Additionally, another group should be included that does not undergo the extinction retention test, which would control for the original memory reactivation and any extinction that might occur.

Implanting cannula might also have altered protein expression, particularly in the IL-mPFC. bFGF has a role in tissue repair (Schultz & Wysocki, 2009), and possibly could have been recruited to the area following surgery. Moreover, scar tissue likely forms around the cannula, which could also alter protein expression in the area. To control for this variable, an additional control group that is treated identically as the other four groups could be included, but that do not receive cannula or microinfusions.

One final limitation to these results is the manner by which the western blots were conducted. Due to limited time and resources, the western blots were conducted simultaneously, with all proteins of interest per structure being examined at once or following numerous strips and reprobes of the membrane. Each brain region was conducted in this manner in immediate succession. Conducting the western blots in this manner likely and greatly increased the risk of human error. Thus, to confirm the results presented here, replication of each protein of interest per structure is warranted.

Extinction of drug seeking alters drug-induced plasticity

Overall, there was some evidence that extinction could attenuate cocaine-induced plastic changes in IL-mPFC, NAC, and dHipp, as rats that underwent extinction had changes in protein expression when compared to naïve or rats that had different length of withdrawal. However, rats that underwent extinction were not different than rats that remained in their home cages throughout, when the length of withdrawal was equal, with the exception of ARC and bFGF in IL-mPFC. Thus, extinction does alter cocaine-induced plastic changes, but reversal was only observed in IL-mPFC with bFGF and ARC protein expression.

These findings are not surprising when reinstatement and relapse is considered. Rats will reliability reinstate drug seeking following a priming injection of cocaine, a stressful event, or following presentation of a cue previously not extinguished (Epstein et al., 2006; Shaham, Shalev, Lu, De Wit, & Stewart, 2003). This is not unique to rats, as most addicts relapse following treatment (McLellan et al., 2000), and extinction-based exposure therapy is not particularly effective (Conklin & Tiffany, 2002). It is thought that prolonged stimulant use induces long-lasting maladaptive changes to the brain, specifically to the reward pathway, that makes abstinence difficult (Hyman, Malenka, & Nestler, 2006; Kalivas, 2009). In conjunction, stimulant drugs are a potent reinforcer that can become associated with cues, which can trigger craving and promote relapse in an abstinent addict (Childress et al., 1986).

Extinction is the formation of a new memory wherein the cues are no longer associated with the reward (Millan et al., 2011; Quirk & Mueller, 2008). However, as

discussed, extinction alone is not sufficient to prevent relapse. Considerable work has been conducted to find pharmacological adjuncts to pair with exposure based extinction therapy (Chesworth & Corbit, 2015), in hopes that if extinction was strengthened, it could prevent subsequent relapse. Progress has been made with opiates and alcohol treatment (Aboujaoude & Salame, 2016; Rodriguez-Arias, Aguilar, & Minarro, 2015), but there is no FDA approved medication for cocaine addiction.

Extinction of drug seeking is less well characterized than reinstatement or relapse. However, in order to strengthen extinction learning, the mechanisms underlying extinction should be determined. If stimulant drug use is promoting a state that makes it difficult to form new memories, then methods to reverse this state should be determined. Therefore, the goal of these experiments was to determine if extinction could alter cocaine-induced plasticity. None of the proteins of interest, except for bFGF in our preliminary data (Hafenbreidel et al., 2015) and ARC in IL-mPFC, reversed cocaine-induced plasticity. However, extinction did alter the expression of ARC, bFGF, GluR1, MAP2, pGSK3β, and PSD95 at either the early or late withdrawal period in at least one brain region. These changes give new directions for ways to being strengthening extinction to ultimately reduce drug seeking and prevent relapse.

The role of bFGF in extinction of drug seeking

The second central question Aim 1 was trying to address was if neutralizing bFGF in IL-mPFC prior to four 30-min extinction sessions facilitated any extinction-induced plastic changes. With the exception of ARC (see above), no other protein of interest was significantly different between groups that received veh- or anti-bFGF

infusions, in any brain region. Thus, at the time point selected, neutralizing bFGF in IL-mPFC did not affect cocaine-induced plasticity.

In addition to the aforementioned limitations, the limited number of extinction-induced changes in cocaine-induced plasticity made any conclusions from these groups difficult. There were no significant changes in bFGF protein expression in IL-mPFC in rats that received infusions of anti-bFGF, which indicates that neutralizing bFGF is not long lasting. Therefore, it is possible that any plastic changes induced by bFGF during the first four 30-min extinction sessions were also reversed by extinction day five. Moreover, learning induced changes may not have been evident either, as animals were euthanized immediately following the retention test, and rats had not fully extinguished drug seeking. Our findings do not explain the differences in behavior observed on extinction day five, but it is possible that western blotting is not sensitive enough to detect the changes that mediated the facilitated extinction observed in anti-bFGF infused rats during the extinction retention test (extinction day five).

A more robust technique to determine the mechanisms underlying the effect of neutralizing bFGF on extinction of drug seeking would be to conduct electrophysiological recordings. bFGF reduces intrinsic excitability when bath applied to hippocampal neurons (Cuppini et al., 2009; Hilborn et al., 1998), and blocks calcium-dependent inactivation of NMDA receptors (Boxer et al., 1999). Therefore, the first step would be to determine if neutralizing bFGF could reverse these effects. Next, stimulant drug use can decrease intrinsic excitability in NAc (Dong et al., 2006; Ishikawa et al., 2009; Kourrich & Thomas, 2009; Mu et al., 2010). Thus, the following step would be to determine if cocaine self-administration also decreased excitability in IL-mPFC and if

neutralizing bFGF could reverse these changes. Finally, the last step would be determining if extinction could also reverse these changes in excitability and if neutralizing bFGF potentiated this reversal.

Another potential way to examine the mechanisms underlying the effect of neutralizing bFGF on extinction would be to systematically block targets down stream from bFGF (Figure 2), either pharmacologically or genetically (e.g., siRNA). One additional approach would be to examine epigenetic modifications following extinction of drug seeking. Epigenetic modifications have emerged as a potent regulator of neuroplasticity in drug addiction (Nestler, 2014). Epigenetic modifications are changes made to the DNA or proteins associated with the DNA (histones) that alter transcription. One type is histone methylation, which is associated with a closed chromatin state and reduced transcription. bFGF is regulated through histone methylation; specifically the expression of H3K9me3 on bFGF is associated with decreased expression of bFGF in the NAc (Chaudhury et al., 2014). However, the extent that H3K9me3 regulates the differential bFGF expression following self-administration or extinction is unknown. Therefore, a future investigation could test the hypothesis that cocaine self-administration decreases H3K9me3 at the bFGF promoter resulting in increased bFGF expression, but the opposite occurs following extinction in IL-mPFC.

AIM 2

RESULTS

To determine the behavioral relevance of our previous findings that indicated that extinction of cocaine seeking also modified bFGF protein expression in the NAc and dHipp, rats were infused with vehicle or anti-bFGF one hour before four 30-min extinction sessions or were immediately returned their home cages without being exposed to the operant chambers. Extinction retention was then tested, after a two-day drug wash out period, during a 90-min extinction session. Finally, the long-term effects of neutralizing bFGF in NAc and dHipp were examined by inducing reinstatement with a priming injection of cocaine.

Neutralizing bFGF in NAc before four 30-min extinction sessions disrupted initial extinction retention

Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 8). Rats infused with vehicle in NAc of dHipp that did undergo 30-min extinction sessions were not significantly different during acquisition (Table 8; active lever presses, all t_{13}=0.875, p=0.397), extinction (Figures 19 and 20; active lever presses, all Fs<3.467, p>0.05), or reinstatement (Figures 19 and 20; active lever presses, $F_{1,24}$=1.879, p=0.183) and therefore were collapsed into a single vehicle group.

Group	Treatment	Ext (30 min)	Cannula	n	Active lever	Inactive lever	Infusions
1	Veh	✓	NAc	7	37.42 ± 9.47	1.29 ± 0.74	27.50 ± 1.90
	Anti-bFGF	✓	NAc	9	37.30 ± 4.19	2.48 ± 1.13	27.15 ± 1.99
2	Veh	X	NAc	8	32.25 ± 2.60	42.92 ± 39.46	27.13 ± 1.54
	Anti-bFGF	X	NAc	8	34.85 ± 4.76	2.37 ± 1.19	26.63 ± 2.47
3	Veh	✓	dHipp	8	32.71 ± 4.61	1.04 ± 0.46	25.46 ± 1.79
	Anti-bFGF	✓	dHipp	9	42.22 ± 13.47	0.81 ± 0.35	25.85 ± 2.28
4	Veh	X	dHipp	7	37.75 ± 5.55	0.58 ± 0.30	25.50 ± 2.08
	Anti-bFGF	X	dHipp	8	41.71 ± 8.32	21.17 ± 17.67	26.21 ± 2.09

Table 8: Average number of active and inactive lever presses, or infusions across the last three days of cocaine self-administration. There were no significant differences between groups.

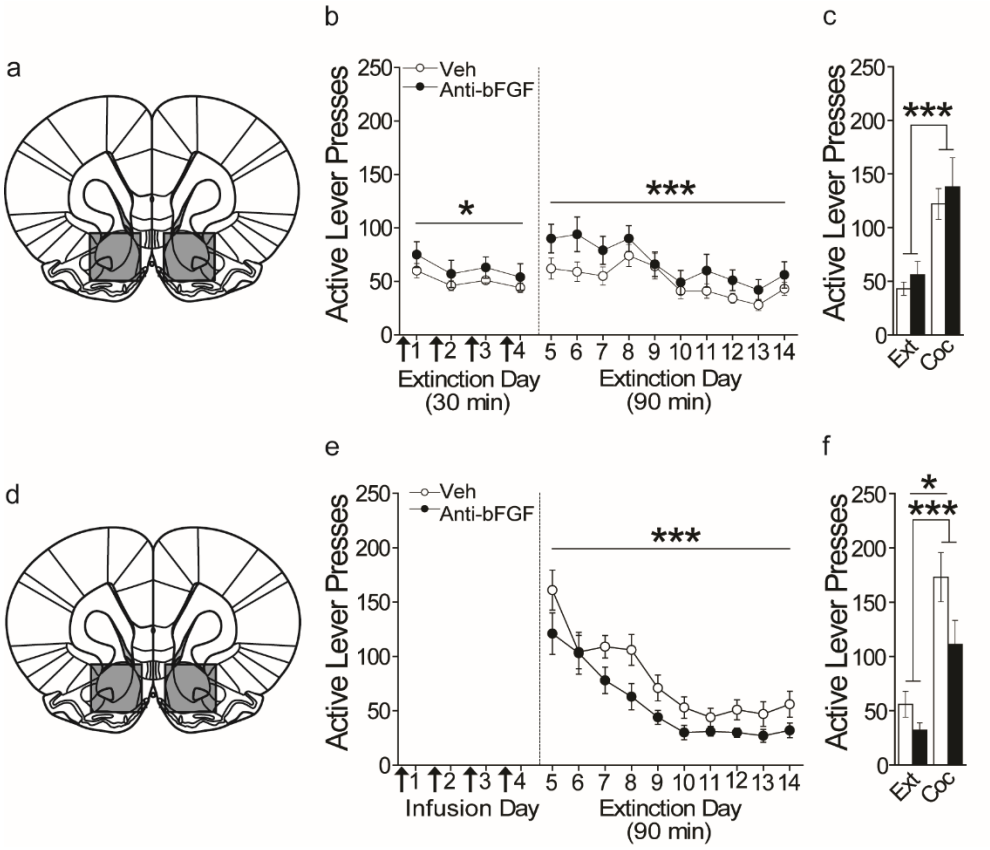

Figure 19: Neutralizing bFGF in NAc before four 30-min extinction sessions disrupts initial extinction, but neutralizing bFGF without extinction facilitates subsequent extinction. (a) Representative area for injector tip locations in NAc. (b) Pre-extinction session infusions of anti-bFGF into NAc (arrows) during the 30-min extinction sessions disrupted extinction retention compared to vehicle infusions. (c) All rats reinstated following a priming injection of cocaine. (d) Representative area for injector tip locations in NAc. (e)

Infusions of either vehicle or anti-bFGF into NAc for four days (arrows), during which rats were returned immediately to their home cages, facilitated subsequent extinction. (f) All rats reinstated following a priming injection of cocaine, but anti-bFGF-infused rats were attenuated. *p<0.05, ***p<0.0001 Error bars are ±SEM.

Following acquisition, rats underwent 38 days of withdrawal in their home cages. Next, rats received vehicle or anti-bFGF infusions into NAc one hour before four 30-min extinction sessions. Across the 30-min extinction sessions (days 1-4, active lever presses; Figure 19b, left), ANOVA revealed a significant effect of treatment ($F_{1,88}$=4.769, p=0.032), but no effect of day ($F_{3,88}$=2.305, p=0.082) or day by treatment interaction ($F_{3,88}$=0.031, p=0.993). During the 90-min extinction sessions (days 5-14; Figure 19b, right), ANOVA revealed a significant effect of treatment ($F_{1,220}$=15.051, p<0.0001), day ($F_{9,220}$=5.187, p<0.0001), but no day by treatment interaction ($F_{9,220}$=0.442, p=0.911). These results indicate that both groups demonstrate little extinction during the first four 30-min extinction days, but anti-bFGF-infused rats lever pressed more. Moreover, anti-bFGF-infused rats lever pressed more during the 90-min extinction sessions, suggesting impaired extinction retention, but both groups demonstrate extinction across days. Inactive lever presses (data not shown) did not differ across days or treatment regardless of extinction session duration (all Fs<3.367, ps>0.05). Thus, neutralizing bFGF in the NAc disrupted initial extinction retention.

Following extinction, all rats were tested for cocaine-induced reinstatement of drug seeking. Rats were given a non-contingent priming injection of cocaine (Figure 19c) prior to a 90-min extinction session. ANOVA revealed a significant increase in active lever pressing during the reinstatement test as compared to the last extinction

test ($F_{1,42}$=28.690, p<0.0001), but no effect of treatment ($F_{1,42}$=0.694, p=0.410) or day by treatment interaction ($F_{1,42}$=0.001, p=0.975). Inactive lever presses did not differ across days or treatment (all Fs<2.504, ps>0.05; data not shown). Therefore, neutralizing bFGF in the NAc during the first four extinction days did not affect cocaine-induced reinstatement.

Neutralizing bFGF in NAc for four days, without extinction training, enhanced extinction retention

Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 8). Rats infused with vehicle in NAc of dHipp that did not undergo 30-min extinction sessions were not significantly different during acquisition (Table 8; active lever presses, all t_{13}=0.881, p=0.394), extinction (Figures 19 and 20; active lever presses, all $F_{1,130}$=0.740, p=0.391), or reinstatement (Figures 19 and 20; active lever presses, $F_{1,26}$=0.224, p=0.640) and therefore were collapsed into a single vehicle group.

Following acquisition, rats underwent 38 days of withdrawal in their home cages. Next, rats received vehicle or anti-bFGF infusions into NAc and were immediately returned to their home cages, without being exposed to the operant chambers, for four days (Figure 19e, left). Next, rats underwent daily 90-min extinction sessions. During the 90-min extinction sessions (days 5-14; Figure 19e, right), ANOVA revealed a significant effect of treatment ($F_{1,210}$=16.597, p<0.0001), day ($F_{9,210}$=14.256, p<0.0001), but no day by treatment interaction ($F_{9,210}$=0.417, p=0.925). These results indicate that

both groups demonstrate extinction across days, but anti-bFGF-infused rats extinguish at a quicker rate than vehicle-infused rats.

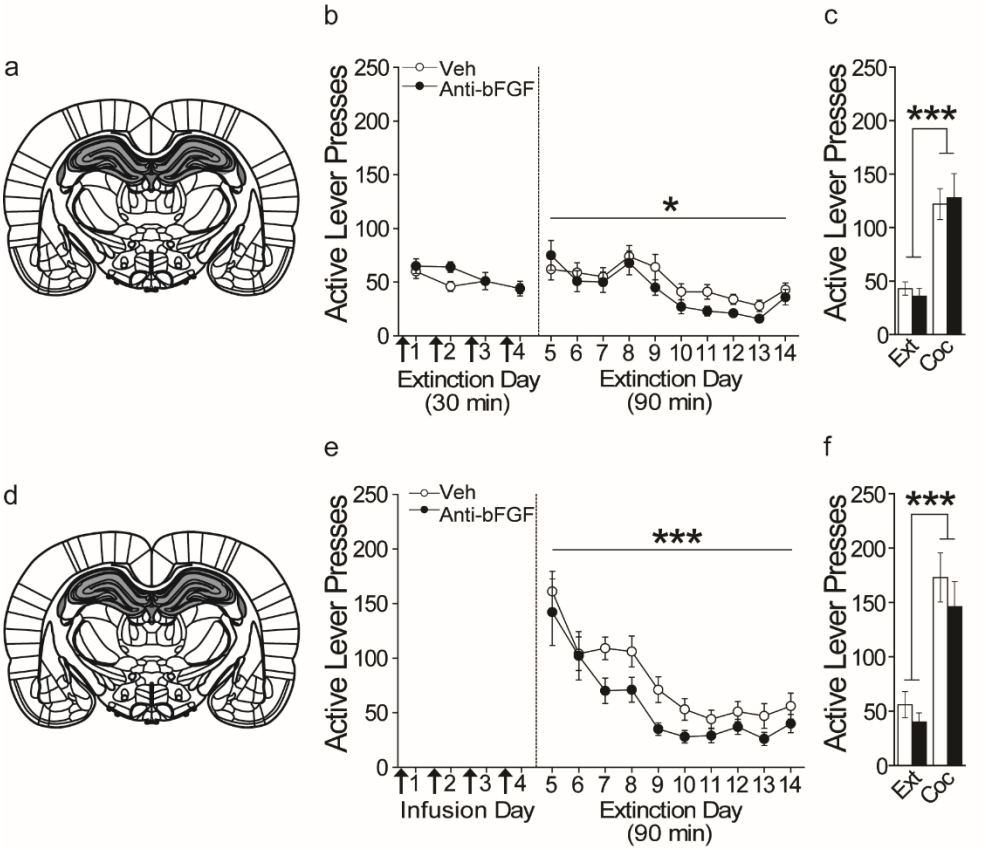

Figure 20: Neutralizing bFGF in dHipp, with or without four 30-min extinction sessions, facilitates subsequent extinction. (a) Representative area for injector tip locations in dHipp. (b) Pre-extinction session infusions of anti-bFGF into dHipp (arrows) during the 30-min extinction sessions facilitates extinction retention compared to vehicle infusions. (c) All rats reinstated following a priming injection of cocaine. (d) Representative area for injector tip locations in dHipp. (e) Infusions of either vehicle or anti-bFGF into dHipp for four days (arrows), during which rats were returned immediately to their home cages, facilitated subsequent extinction. (f) All rats reinstated following a priming injection of cocaine. *p<0.05, ***p<0.0001 Error bars are ±SEM.

For inactive lever presses during extinction days 5-14 (data not shown), ANOVA revealed a significant effect of treatment ($F_{1,210}$=8.971, p=0.003), day ($F_{9,210}$=2.285, p=0.018), but no day by treatment interaction ($F_{9,210}$=0.340, p=0.961). A subsequent ANOVA between active and inactive lever presses revealed a significant effect of lever ($F_{1,420}$=430.918, p<0.0001), which demonstrates that rats pressed the active lever more than the inactive lever. Finally, veh-infused rats that underwent four 30-min extinction sessions lever pressed significantly less than veh-infused rats that did not undergo the four 30-min extinction sessions during extinction day five (t_{13}=-3.635, p=0.003; data not shown), which indicates that four 30-min extinction sessions are sufficient for extinction learning to occur. In summary, neutralizing bFGF in the NAc, without four 30-min extinction sessions, facilitated subsequent extinction across days.

Following extinction, all rats were tested for cocaine-induced reinstatement of drug seeking. Rats were given a non-contingent priming injection of cocaine (Figure 19f) prior to a 90-min extinction session. ANOVA revealed a significant increase in active lever pressing during the reinstatement test as compared to the last extinction test ($F_{1,42}$=24.980, p<0.0001), and a significant effect of treatment ($F_{1,42}$=4.826, p=0.034), but no day by treatment interaction ($F_{1,42}$=0.984, p=0.327). Inactive lever presses did not differ across days or treatment (all *Fs<1.153, ps*>0.05). In summary, previously veh- or anti-bFGF-infused rats reinstated cocaine seeking following a priming injection of cocaine, however, previously anti-bFGF-infused rats appear to have attenuated seeking. These results suggest that neutralizing bFGF in the NAc, without 30-min extinction sessions, may help protect against cocaine-induced reinstatement.

Neutralizing bFGF in dHipp before four 30-min extinction sessions facilitates extinction

Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 8). Following acquisition, rats underwent 38 days of withdrawal in their home cages. Next, rats received vehicle or anti-bFGF infusions into dHipp one hour before four 30-min extinction sessions. Across the 30-min extinction sessions (days 1-4, active lever presses; Figure 20b, left), ANOVA revealed a significant effect of day ($F_{3,88}$=3.764, p=0.014), but no effect of treatment ($F_{1,88}$=2.108, p=0.150) or day by treatment interaction ($F_{3,88}$=1.132, p=0.341). During the 90-min extinction sessions (days 5-14; Figure 20b, right), ANOVA revealed a significant effect of treatment ($F_{1,230}$=4.903, p=0.028), day ($F_{9,220}$=7.476, p<0.0001), but no day by treatment interaction ($F_{9,220}$=0.542, p=0.843). These results indicate that both groups reduced lever pressing during the first four 30-min extinction and during the 90-min extinction days indicating both groups demonstrated extinction across days, but anti-bFGF-infused rats extinguish at a quicker rate than vehicle-infused rats during the 90-min extinction days. Inactive lever presses (data not shown) did not differ across days regardless of extinction session duration (all Fs<1.443, ps>0.05), but ANOVA did reveal a significant treatment effect (extinction days 1-4: $F_{1,88}$=4.498, p=0.037; extinction days 5-15: $F_{1,220}$=14.759, p<0.0001). Subsequent ANOVAs between active and inactive levers demonstrated a significant effect of lever (extinction days 1-4: $F_{1,176}$=521.859, p<0.0001; extinction days 5-15: $F_{1,440}$=456.583, p<0.0001), which demonstrates that rats pressed significantly

more on the active lever than the inactive. Overall, neutralizing bFGF in the dHipp before four 30-min extinction sessions facilitated subsequent extinction across days.

Following extinction, all rats were tested for cocaine-induced reinstatement of drug seeking. Rats were given a non-contingent priming injection of cocaine (Figure 20c) prior to a 90-min extinction session. ANOVA revealed a significant increase in active lever pressing during the reinstatement test as compared to the last extinction test ($F_{1,42}$=40.724, p<0.0001), but no effect of treatment ($F_{1,42}$=0.032, p=0.859) or day by treatment interaction ($F_{1,42}$=0.099, p=0.754). Inactive lever presses did not differ across days or treatment (all Fs<0.891, ps>0.05). Therefore, neutralizing bFGF in the dHipp during the first four extinction days did not affect cocaine-induced reinstatement.

Neutralizing bFGF in dHipp for four days, without extinction training, facilitates subsequent extinction across days

Active or inactive lever presses and number of infusions were equivalent between groups across the average of the last three days of self-administration (Table 8). Following acquisition, rats underwent 38 days of withdrawal in their home cages. Next, rats received vehicle or anti-bFGF infusions into dHipp and were immediately returned to their home cages, without being exposed to the operant chambers, for four days. Next, rats underwent daily 90-min extinction sessions. During the 90-min extinction sessions (days 5-14), ANOVA revealed a significant effect of treatment ($F_{1,210}$=12.406, p<0.0001), day ($F_{9,210}$=14.546, p<0.0001), but no day by treatment interaction ($F_{9,210}$=0.347, p=0.958). These results indicate that both groups demonstrate extinction across days, but anti-bFGF-infused rats extinguish at a quicker

rate than vehicle-infused rats. For inactive lever presses during extinction days 5-14 (data not shown), ANOVA revealed a significant effect of day ($F_{9,210}$=2.930, p=0.003), but no effect of day or day by treatment interaction (all Fs<2.046, ps>0.05). Finally, veh-infused rats that underwent four 30-min extinction sessions lever pressed significantly less than veh-infused rats that did not undergo the four 30-min extinction sessions during extinction day five (t_{13}=-3.069, p=0.009; data not shown), which indicates that four 30-min extinction sessions are sufficient for extinction learning to occur. Overall, neutralizing bFGF in the dHipp, without four 30-min extinction sessions, facilitated subsequent extinction across days.

Following extinction, all rats were tested for cocaine-induced reinstatement of drug seeking. Rats were given a non-contingent priming injection of cocaine (Figure 20f) prior to a 90-min extinction session. ANOVA revealed a significant increase in active lever pressing during the reinstatement test as compared to the last extinction test ($F_{1,42}$=31.706, p<0.0001), but no effect of treatment ($F_{1,42}$=1.153, p=0.289) or day by treatment interaction ($F_{1,42}$=0.081, p=0.778). Inactive lever presses did not differ across days or treatment (all *Fs<2.317, ps*>0.05). Therefore, neutralizing bFGF in the dHipp for four days, without 30-min extinction sessions, did not affect cocaine-induced reinstatement.

Infusing bFGF in IL-mPFC was not sufficient to disrupt extinction of sucrose seeking

We previously found that neutralizing bFGF in IL-mPFC facilitates extinction of cocaine seeking (Hafenbreidel et al., 2015), however, if overexpression of bFGF in IL-

mPFC was sufficient to prolong extinction was unknown. Extinction of sucrose seeking does not alter bFGF expression in IL-mPFC (Hafenbreidel et al., 2015), so infusing bFGF into this region would result in overexpression. Thus, to determine if overexpression of bFGF in IL-mPFC is sufficient to disrupt extinction, rats received infusions of bFGF one hour before four 90-min extinction of sucrose seeking sessions.

Active lever presses were equivalent between groups across the average of the last three days of sucrose self-administration ($t(21)=-0.001$, $p=0.999$). Following acquisition, rats received vehicle or bFGF infusions into IL-mPFC one hour before four 90-min extinction sessions. Across the 90-min sessions (days 1-8, active lever presses; Figure 21b), ANOVA revealed a significant effect of day ($F_{7,168}=57.160$, $p<0.0001$), but no significant effect of treatment ($F_{1,168}=1.056$, $p=0.306$) or day by treatment interaction ($F_{7,168}=0.413$, $p=0.893$). Overall, these results demonstrate that brief bFGF overexpression prior to extinction training is not sufficient to prolong extinction of sucrose seeking.

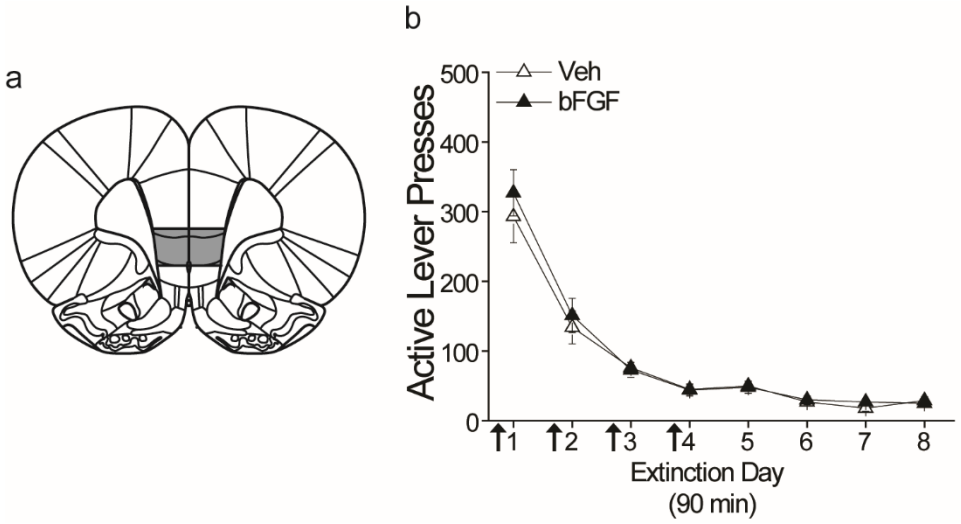

Figure 21: Infusing bFGF in IL-mPFC is not sufficient to disrupt extinction of sucrose seeking, (a) Representative area for injector tip locations in IL-mPFC. (b) Pre-extinction session infusions of bFGF into IL-mPFC (arrows) did not affect extinction. Error bars are ±SEM.

Neutralizing bFGF in IL-mpFC and dHipp does not affect locomotion, but does in NAc

To determine if neutralizing bFGF in IL-mPFC, NAc, or dHipp affected general motor activity, rats were infused with vehicle or anti-bFGF one hour prior to being placed in locomotor activity chambers for 20 minutes (Figure 22). No difference in photobeam breaks between groups in IL-mPFC (t_5=0.565, p=0.597) or dHipp (t_{14}=-0.869, p=0.400) were observed, demonstrating that locomotor activity was not affected by neutralizing bFGF. However, neutralizing bFGF in NAc significantly increased locomotion (t_{13}=2.649, p=0.020), demonstrating a region specific effect of anti-bFGF on locomotion. This increase in general motor activity may explain the increase in lever pressing observed in rats infused with anti-bFGF that did receive 30-min extinction sessions that was not observed in anti-bFGF-infused rats that did not receive 30-min sessions. To control for this effect, anti-bFGF could be infused immediately after the 30-min session, and this would also determine if neutralizing bFGF affects extinction acquisition or extinction consolidation.

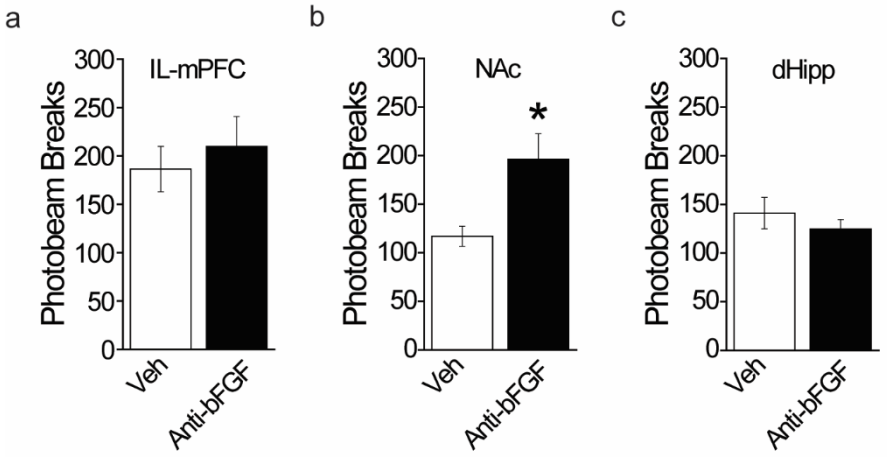

Figure 22: Neutralizing bFGF in IL-mPFC and dHipp does not affect locomotion, but does in NAc. (a) Neutralizing bFGF in IL-mPFC does not affect locomotion. (b) Neutralizing bFGF in NAc increases locomotion. (c) Neutralizing bFGF in dHipp does not affect locomotion. *p<0.05 Error bars are ±SEM.

Cannula placement does not affect drug seeking, but withdrawal duration does

To determine if lowering cannula into specific brain regions affected drug seeking following acquisition, an additional analysis was conducted on the number of active lever presses made during the first 90-min extinction session. Additionally, to determine if our particular acquisition paradigm induced potentiated drug seeking following a withdrawal period, the number of lever presses made during the first 90-min extinction session was examined in rats that underwent either a short or long withdrawal period. Rats that underwent 30-min extinction sessions were excluded from this analysis, and the number of withdrawal days indicates the number of days that elapsed between the final day of acquisition and the first 90-min extinction day. First, rats that were not implanted with cannula, but did undergo different number of withdrawal days were compared (Figure 23a). A one-way ANOVA revealed a significant difference in active lever pressing between rats that underwent three, 11, 38, or 50 withdrawal days ($F_{3,38}$=4.790, p=0.006). Post hoc analysis confirmed that rats that underwent 38 days of withdrawal lever pressed significantly more than rats that underwent three (p=0.014) or 11 (p=0.024) days of withdrawal, but were not significantly different than rats that underwent 50 days of withdrawal (p=0.854). Likewise, rats that underwent three or 11 days of withdrawal lever pressed similarly (p=0.800). Finally, rats that underwent 50 days of withdrawal lever pressed significantly more than rats that underwent three (p=0.005) or 11 days of withdrawal (p=0.010). These results demonstrate that drug seeking is potentiated after a prolonged withdrawal period after acquisition when using our specific parameters (i.e., 18 days, 90 min a day, 4-5 days in a row followed by 2-3 day breaks).

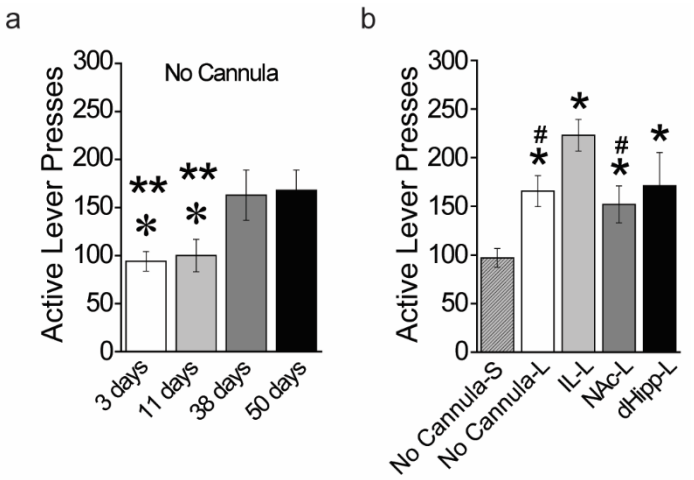

Figure 23: Drug seeking is potentiated after a prolonged withdrawal period. (a) Rats lever press significant more during the first 90-min extinction session after a prolonged withdrawal period. (b) Rats with IL-mPFC cannula lever press more than rats with no cannula or NAc cannula, but all rats that underwent a prolonged withdrawal period (L) lever pressed more than rats that underwent a short withdrawal period (S). ** $p<0.001$ compared to 50 days, * $p<0.05$ compared to 38 days, * $p<0.05$ compared to No cannula-S, # $p<0.05$ compared to IL-L Error bars are ±SEM.

Next, because rats that underwent three or 11 days of withdrawal were not significantly different, they were collapsed into a single "short" withdrawal group (whole-S). Similarly, rats that underwent 38 or 50 days of withdrawal were collapsed into a single "long" withdrawal group (whole-L) as they were not significantly different. These two groups were then compared to rats that received IL-mPFC, NAc, or dHipp cannula that also underwent 38-days of withdrawal (IL-L, NAc-L, dHipp-L, respectively; Figure 23b). A one-way ANOVA revealed a significant difference in active lever pressing between groups ($F_{4,63}=9.190$, $p<0.0001$). Post hoc analysis confirmed that whole-S rats lever pressed significantly less than whole-L rats ($p=0.001$), IL-L rats ($p<0.0001$), NAc-L rats ($p=0.030$), and dHipp-L rats ($p=0.006$). These results indicate that regardless of cannula placement or no cannula, a long withdrawal period will potentiate drug seeking following self-administration. However, post hoc analysis also revealed that IL-L rats lever pressed significantly more than whole-L rats ($p=0.016$) and NAc-L rats ($p=0.013$),

but not dHipp-L rats (p=0.078). Overall, these results suggest that a long withdrawal period will result in potentiated drug seeking, but cannula placement may also affect drug seeking.

Discussion

The current experiments aimed to determine the functional relevance of our preliminary findings that extinction altered bFGF protein expression in the NAc and dHipp. We found that neutralizing bFGF in NAc before four 30-min extinction sessions disrupted extinction retention, initially. However, when bFGF was neutralized in NAc for four days, without rats undergoing four 30-min extinction sessions, subsequent extinction was facilitated and cocaine-induced reinstatement was attenuated. Moreover, neutralizing bFGF in dHipp, with or without 30-min extinction sessions, facilitated subsequent extinction. Finally, we found that infusing bFGF into IL-mPFC, to mimic drug-induced overexpression, before four 90-min extinction of sucrose seeking sessions was not sufficient to prolong extinction.

We previously found that neutralizing bFGF in IL-mPFC before four 30-min extinction sessions was necessary for facilitated extinction retention, as rats pressed similarly throughout extinction when they were infused without undergoing four 30-min extinction sessions (Figure 10). Alternatively, neutralizing bFGF in NAc only facilitated extinction when rats did not received four 30-min extinction sessions. Moreover, neutralizing bFGF in dHipp facilitated extinction with or without the four 30 min sessions. These results suggest that neutralizing bFGF without extinction is sufficient to enhance subsequent extinction in NAc and dHipp. Rats lever press similarly on the first 90-min extinction sessions, but the rate by which anti-bFGF-infused rats reduce lever pressing across days is quicker than vehicle-infused rats. These results indicate that neutralizing bFGF does not simply impair the rat's ability to retrieve the original drug-cue memory or supplant an extinction memory, but instead that they are able to learn extinction at a

faster rate than vehicle-infused rats over days. Moreover, in the NAc, neutralizing bFGF without four 30-min extinction sessions appears to have attenuated cocaine-induced reinstatement.

The use of the shortened extinction sessions allows for the possibility that the rat does not have sufficient time to begin learning the new contingencies that the cues are no longer associated with drug administration, and thus would serve only as a memory reactivation session. If that were the case, then the reduction of lever pressing by anti-bFGF-infused rats would be due to either retrieval or reconsolidation disruption, because bFGF is neutralized before the session, and not due to enhanced extinction acquisition or consolidation. However, because neutralizing bFGF in NAc and dHipp without undergo four 30 min extinction sessions resulted in facilitated rate of subsequent extinction, it does not seem likely that the original drug-cue memory was affected. Moreover, veh-infused rats that underwent the four 30-min extinction sessions lever press less than veh-infused rats that did not undergo extinction during the first 90-min extinction session (IL-mPFC, NAc, and dHipp), which demonstrates that the four 30 min extinction sessions were sufficient for rats to learn extinction. Overall, these results suggest that neutralizing bFGF is affecting extinction, and not the retrieval or reconsolidation of the original drug-cue memory.

Finally, it is interesting that opposite effects were observed in the NAc depending on whether or not rats underwent 30-min extinction sessions. A possible explanation for these contrasting results is that a drug-state effect may have affected behavior during the 30-min extinction sessions. We found that infusing vehicle or anti-bFGF into IL-

mPFC or dHipp one hour before locomotion testing did not alter general motor activity, but infusing anti-bFGF into NAc did increased general motor activity.

The diverse role of bFGF in learning and plasticity

One possible explanation for our results is that blocking the biological function of bFGF primes the region for subsequent plasticity, such as what is necessary for new learning. As discussed previously, stimulant drug administration can induce a number of structural and functional changes in reward related brain regions. Particularly, stimulant drug administration decreases intrinsic excitability in the NAc (Dong et al., 2006; Ishikawa et al., 2009; Kourrich & Thomas, 2009; Mu et al., 2010). The effect of bFGF application on intrinsic excitability in NAc has not been determined, but bFGF reduces intrinsic excitability in dHipp (Cuppini et al., 2009). Thus, when bFGF was neutralized, this could limit its effect on excitability, perhaps allowing for subsequent new plasticity and learning to occur, such as extinction. In agreement with this, bFGF protein expression was increased in Coc NE(38) rats in dHipp (Figure 18c), and there was a trend for increased expression in IL-mPFC (Figure 14b) and NAc (Figure 17c), but blocking bFGF expression in any of these brain regions, under certain parameters, can enhance subsequent extinction.

These results are interesting considering that infusing bFGF into the amygdala can enhance extinction of conditioned fear (Graham & Richardson, 2011a, 2011b). It is unclear if this is a region specific effect, or if bFGF has unique roles in extinction of drug seeking and extinction of conditioned fear. It is unknown if fear conditioning alters bFGF protein expression, but increased bFGF is associated with lower anxiety-related

behaviors and decreased bFGF is associated with higher anxiety-related behaviors in selectively bred ratlines (Graham & Richardson, 2011b; Riva et al., 2005). Fear conditioning can induce anxiety, and thus may decrease bFGF expression, but this has not been tested yet. bFGF may function optimally in a very narrow and specific range. Thus, when expression is decreased by an anxiety-provoking event, perhaps like fear conditioning, then infusions of bFGF would increase it back into its optimal range and allow for enhanced learning. Alternatively, when expression is increased by stimulant drug administration, then infusions of anti-bFGF would reduce it back into its optimal range and allow for enhanced learning.

In agreement with this notion, bFGF also has a role in glutamate signaling. Besides inhibiting NMDA receptors (Boxer et al., 1999), bFGF can induce glutamate release through interactions with voltage-gated calcium channels and the MAPK pathway in culture neurons (Graham & Richardson, 2011b; Numakawa et al., 2002). Increased glutamate could explain the enhancement in learning, and NMDA receptor activation is necessary for learning in a number of paradigms (Fiorenza, Rosa, Izquierdo, & Myskiw, 2012; Hafenbreidel et al., 2014; Hammond, Seymour, Burger, & Wagner, 2012; Kelamangalath, Seymour, & Wagner, 2009; Langton & Richardson, 2008; Nic Dhonnchadha et al., 2010).

However, in disagreement, we found that infusing bFGF into IL-mPFC before four extinction of sucrose seeking sessions had no effect on the rate of extinction. The dose used was based off of previous research (Graham & Richardson, 2011a; Turner et al., 2009; Wagner et al., 1999), but the dose may not have been high enough to match the increase observed following stimulant drug administration. Another possibility is that

cocaine-induced overexpression does not induce maladaptive changes quickly, but instead requires prolonged over expression.

The role of bFGF in extinction of drug seeking circuitry

Overall, we found that neutralizing bFGF in IL-mPFC before four 30-min extinction sessions facilitated extinction retention, neutralizing bFGF in NAc for four days without extinction facilitated subsequent extinction, and neutralizing bFGF in dHipp before four 30-min extinction sessions or without extinction facilitated subsequent extinction. The most robust finding was in the IL-mpFC, which agrees with previous research that it is necessary for extinction (LaLumiere et al., 2010; Peters et al., 2008). The IL-mPFC sends projections to the NAc shell (Groenewegen et al., 1999; Sesack et al., 1989), which is necessary for inhibiting drug seeking following extinction, but if it is necessary for extinction acquisition is unclear (Peters et al., 2009; Peters et al., 2008). The NAc is thought to regulate the motor outputs that drive drug seeking (Peters et al., 2009), which may explain why this was the only brain region that had increased locomotion following infusions of anti-bFGF, and had attenuated cocaine-induced reinstatement.

The dorsal hippocampus may not have any direct projections to the IL-mPFC or NAc, but does have indirect projections, such as one projection to the NAc core through the vHipp (Figure 1; Fanselow & Dong, 2010; Groenewegen, Wright, & Beijer, 1996; Naber & Witter, 1998). It is not clear what role the vHipp may have in extinction of drug seeking. We previously found that rats that did not undergo extinction had reduced bFGF immunoreactivity in vHipp compared to naïve rats, which was slightly attenuated

in rats that did undergo extinction (Figure 9). However, if this brain region, or some other region, is a critical link between the NAc and dHipp during extinction of drug seeking is unknown. Future experiments should assess the role of the vHipp during extinction, but also the role that bFGF may have in that region during extinction or withdrawal from cocaine self-administration.

FUTURE DIRECTIONS

In the current experiments, we demonstrate that extinction of drug seeking attenuates some plastic changes induced by chronic cocaine use. Moreover, we demonstrate that neutralizing bFGF in NAc and dHipp can facilitate subsequent extinction of drug seeking, and neutralizing bFGF in NAc can attenuate cocaine-induced reinstatement. These results provide new insights to the mechanisms underlying extinction of drug seeking, which provide new directions for future research examining how to strengthen extinction learning in order to reduce relapse rates.

Extinction of drug seeking is not well characterized, and these experiments provided some of the first insights into how extinction, itself, could alter cocaine-induced plastic changes in IL-mPFC, NAc, and dHipp. These results suggest new targets to examine for their behavioral relevance in extinction, or in the case of ARC, possibly in retrieval of the original drug-cue memory. However, as mentioned, the mechanisms underlying the effect of neutralizing bFGF on extinction are still unclear. As discussed, electrophysiological recordings or examining epigenetic modification may begin to explain how neutralizing bFGF in IL-mPFC, NAc, and dHipp results in facilitated extinction.

bFGF could be affecting learning and memory through a number of mechanisms (Graham & Richardson, 2011b), including those suggested in Figure 1. Another possible mechanism could be through its subsidiary interactions with NMDA receptors and the growth factor BDNF (brain derived neurotrophic factor). bFGF has opposing effects on extinction compared to BDNF. An infusion of BDNF into IL-mPFC facilitates extinction (Otis et al., 2014; Peters, Dieppa-Perea, Melendez, & Quirk, 2010; Xin et al.,

2014) and endogenous BDNF mRNA expression increases in IL-mPFC during extinction of a conditioned taste aversion (Xin et al., 2014). Furthermore, reducing BDNF activity in the basolateral amygdala and dHipp disrupts learning and extinction (Heldt, Stanek, Chhatwal, & Ressler, 2007; Sakata et al., 2013). In contrast, our results show that endogenous bFGF is reduced in IL-mPFC following extinction and blocking bFGF in this region facilitates extinction, suggesting that BDNF and bFGF have inverse roles during extinction. These opposing roles could be mediated by interactions with NMDA receptors. For example, BDNF facilitates extinction of drug seeking through its interaction with NMDA receptors (Otis et al., 2014). However, bFGF diminishes the function of these receptors (Boxer et al., 1999). NMDA receptor activation is necessary for learning in a number of paradigms (Fiorenza et al., 2012; Hafenbreidel et al., 2014; Hammond et al., 2012; Kelamangalath et al., 2009; Langton & Richardson, 2008; Nic Dhonnchadha et al., 2010). Therefore, future experiments should determine the interaction between NMDA receptors and bFGF, and how they mediate extinction.

Conclusions

Drug addiction is a complex disorder characterized by compulsive drug seeking, increased impulsivity, and chronic relapse. Nearly 25 million people are reported as needing treatment for drug abuse in the United States (Substance Abuse and Mental Health Services Administration, 2014), and of those that receive treatment, 30-60% relapse within one year (McLellan et al., 2000). The altered behavior and resistance to long-term treatment suggests persistent plastic changes, and these are evident in such instances as modified dendritic and spine morphology and density (Brown & Kolb, 2001; Kolb et al., 2003; Y. Li et al., 2003; Mueller et al., 2006; Robinson & Kolb, 1997, 1999, 2004), and altered expression of molecular markers for neuronal structure and function in reward-related brain regions (e.g., Kauer & Malenka, 2007; Kiraly, Eipper-Mains, et al., 2010; Kourrich et al., 2015; Robison & Nestler, 2011; Wolf, 2010). Determining the mechanisms that underlie each of the characteristics of addiction, and how they interact, is essential to developing new therapeutic strategies.

Drug addiction is also a disorder of learning, as cues become associated with the rewarding properties of the drug. These cues can later trigger craving and withdrawal-like symptoms, which promotes relapse (Childress et al., 1986). Reducing the salience of these cues would therefore reduce the induction of craving and the likelihood of relapse. Alone, however, extinction-based exposure therapy has had limited success (Conklin & Tiffany, 2002). This may be due to another long-term effect of drug use, which is a reduced ability for subsequent experience-dependent plasticity (Kolb et al., 2003) and learning (Divac, 1971; Radley et al., 2015; Sillivan et al., 2011; Skelton et al., 2007; Vorhees et al., 1994; Williams et al., 2002). Therefore, determining the plasticity-

related changes underlying extinction and if they can ameliorate the changes induced by drug use, would allow new targets to be modified, resulting in enhanced learning and better long-term treatment results.

Finally, based off previous work and our preliminary findings, the growth factor bFGF appears to be a key link between drug-induced maladaptive changes and resistance to extinction. bFGF mediates changes in axonal and dendritic morphology (Kalil et al., 2000; Patel & McNamara, 1995; Zechel et al., 2009), intrinsic excitability (Cuppini et al., 2009; Hilborn et al., 1998), is neuroprotective (Anderson et al., 1988; Logan et al., 1992; Mark et al., 1997; Flores and Stewart, 2000a), and contributes to drug-associated behavioral and plastic changes (Flores et al., 2000; Mueller et al., 2006; Turner et al., 2009). Thus, determining the interactions between bFGF, drug-induced plastic changes, and extinction could help identify new targets for pharmacotherapeutrics to enhance treatment for drug addiction.

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Madalyn Hafenbreidel

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EDUCATION:

Bachelor of Arts	Psychology, University of Minnesota-Twin Cities, May 2010
Master of Science	Psychology, University of Wisconsin-Milwaukee, May 2013
Doctorate of Philosophy	Psychology, University of Wisconsin-Milwaukee, August 2016

Undergraduate GPA: 3.636/4.0
Graduate GPA: 3.858/4.0

PUBLICATONS:

Hafenbreidel, M., Twining, R. C., Rafa Todd, C., & Mueller, D. (2015). Blocking infralimbic basic fibroblast growth factor (bFGF or FGF2) facilitates extinction of drug seeking after cocaine self-administration. *Neuropsychopharmacology, 40*(13), 2907-2915.

Hafenbreidel, M., Rafa Todd, C., Twining, R. C., Tuscher, J.J., & Mueller, D. (2014). Bidirectional effects of inhibiting or potentiating NMDA receptors on extinction after cocaine self-administration in rats. *Psychopharmacology (Berl), 231*(24), 4585-4594.

IN PROGRESS PUBLICATONS:

Hafenbreidel, M., Rafa Todd, C., & Mueller, D. Infralimbic GluN2A-containing NMDA receptors mediate reconsolidation of a cocaine self-administration memory (*under review*).

Mechanisms underlying the facilitation of extinction by 17β-estradiol in a cocaine condition place preference paradigm (*in preparation*).

POSTER PRESENTATIONS/ABSTRACTS:

Hafenbreidel, M., Smies, C.W., & Mueller, D. (2016, November). Expression of basic fibroblast growth factor (bFGF or FGF2) is regulated by extinction following cocaine self-administration. Poster to be presented at the upcoming annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, San Diego, CA.

Hafenbreidel, M., Rafa Todd, C., Smies, C.W., Twining, R. C., & Mueller, D. (2015, October). Blocking infralimbic basic fibroblast growth factor (bFGF or FGF2) facilitates extinction of drug seeking. Poster presented at the annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, Chicago, IL.

Hafenbreidel, M., Rafa Todd, C., Smies, C.W., Twining, R. C., & Mueller, D. (2015, September). Blocking infralimbic basic fibroblast growth factor (bFGF or FGF2) facilitates extinction of drug seeking. Poster presented at the meeting of Pavlovian Society, Portland, OR.

Hafenbreidel, M., Rafa Todd, C., Smies, C.W., Twining, R. C., & Mueller, D. (2015, June). Blocking infralimbic basic fibroblast growth factor (bFGF or FGF2) facilitates extinction of drug seeking. Poster presented at the Rat Genetics and Genomics for Psychiatric Disorders and Addiction workshop, Chicago, IL.

Hafenbreidel, M., Rafa Todd, C., Otis, J.M., Twining, R. C., & Mueller, D. (2014, November). Infralimbic NR2A-containing NMDA receptors are necessary for the reconsolidation of cocaine self-administration memory. Poster presented at the annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, Washington, DC.

Hafenbreidel, M., Rafa Todd, C., Twining, R. C., & Mueller, D. (2013, November). NMDA receptors mediate extinction of cocaine self-administration, and enhancing receptor function facilitates extinction. Poster presented at the annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, San Diego, CA.

Hafenbreidel, M., Rafa Todd, C., Twining, R. C., & Mueller, D. (2013, May). NMDA receptors bidirectionally mediate extinction of cocaine self-administration. Poster presented at the meeting of Milwaukee Society for Neuroscience, University of Wisconsin-Milwaukee, Milwaukee, WI.

Hafenbreidel, M., Twining, R. C., & Mueller, D. (2012, October). Neutralizing bFGF in the infralimbic medial prefrontal cortex facilitates extinction of cocaine self-administration. Poster presented at the annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, New Orleans, LA.

Hafenbreidel, M., Twining, R. C., Schneider, J., & Mueller, D. (2011, November). NMDA and β-adrenergic receptor antagonists impair extinction of cocaine seeking. Poster presented at the annual meeting of Society for Neuroscience and meeting of Molecular and Cellular Cognition Society, Washington, DC.

Hafenbreidel, M., Twining, R. C., Schneider, J., & Mueller, D. (2011, September). NMDA and β-adrenergic receptor antagonists impair extinction of cocaine seeking. Poster presented at the meeting of Pavlovian Society, Milwaukee, WI.

Hafenbreidel, M., Twining, R. C., Schneider, J., & Mueller, D. (2011, May). NMDA and β-adrenergic receptor antagonists impair extinction of cocaine seeking. Poster presented at the meeting of Milwaukee Society for Neuroscience, Medical College of Wisconsin, Milwaukee WI.

ORAL PRESENTATIONS:

Mechanisms underlying the role of bFGF (FGF2) in extinction of drug seeking. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, April 8, 2016.

Infralimbic NR2A-containing NMDA receptors are necessary for the reconsolidation of cocaine self-administration memory. Abstract was selected of three from all abstracts submitted to present an oral research talk at UWM-Neuroscience Mini-symposium, a collaboration between Department of Biological Sciences and Department of Psychology, University of Wisconsin- Milwaukee, Milwaukee, WI, March 11, 2016.

The role of NR2A-containing NMDA receptors in cocaine-associated learning. Oral presentation at Department of Psychology Neuroscience Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, November 20, 2015.

Reduction in infralimbic bFGF (FGF2) facilitates extinction of drug seeking. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, April 10, 2015.

Reduction of bFGF (FGF2) facilitates extinction of drug seeking. Oral presentation at Department of Biological Sciences and Department of Psychology Neuroscience Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, February 6, 2015.

The role of NR2A-containing NMDA receptors in cocaine self-administration. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, April 4, 2014.

The role of NR2A-containing NMDA receptors in cocaine self-administration. Oral presentation at Department of Biological Sciences and Department of Psychology Neuroscience Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, March 14, 2014.

The role of NMDA receptors in extinction of cocaine self-administration. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, April 5, 2013.

The role of NMDA receptors in extinction of cocaine self-administration. Oral presentation at Department of Biological Sciences and Department of Psychology Neuroscience Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, February 1, 2013.

bFGF and extinction of cocaine self-administration. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin-Milwaukee, Milwaukee, WI, April 20, 2012.

Glutamate and extinction of cocaine self-administration: Mechanisms and caveats. Oral presentation at Department of Biological Sciences and Department of Psychology Neuroscience Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, March 16, 2012.

NMDA and β-adrenergic receptors antagonist disrupt extinction of cocaine seeking. Oral presentation at the Association for Graduate Students in Psychology Annual Seminar, University of Wisconsin- Milwaukee, Milwaukee, WI, April 8, 2011.

HONORS, AWARDS, AND GRANTS:

- UWM Department of Psychology Summer Research Fellowship (2015).
- UWM Student Association Travel Grant to the annual meeting for Society for Neuroscience in New Orleans, LA (2012), San Diego, CA (2013), Washington, DC (2015), Chicago IL (2015), and San Diego, CA (2016).
- Featured in the Spring 2014 issue of PsychNews, a newsletter for the undergraduate psychology program.
- 2nd place award for data presentation at the Annual Association of Graduate Students in Psychology Seminar (2013).
- Graduate School Graduate Student Travel Award, University of Wisconsin-Milwaukee, Fall 2011, Fall 2012, Fall 2013.
- Lucky Waller Scholarship, University of Minnesota-Twin Cities, 2008-2009.
- Vivian V. Volstorff Scholarship, South Dakota State University, 2006-2007.
- Opportunity Scholarship, South Dakota State University, 2006-2007.
- University of Minnesota-Twin cities Dean's List, 2007-2008; 2010.
- South Dakota State University Dean's List, 2006-2007.

RESEARCH EXPERIENCE:

June 2010-Current: **Graduate research assistant**, Psychology department, UWM. Faculty advisor: Dr. Devin Mueller.
Master's Thesis: The role of NMDA receptors in extinction of cocaine self-administration (2013)
Ph.D. Thesis: The mechanisms underlying cocaine-induced over-expression of basic fibroblast growth factor (bFGF, FGF2), an effect reversed by extinction (2016)

August 2009-May 2010: **Undergraduate research assistant**, Psychology department, University of Minnesota-Twin Cities. Graduate supervisor: Yanna Weisberg. Faculty advisor: Dr. Colin DeYoung.

January 2009-May 2009: **Undergraduate research assistant**, Psychology department, University of Minnesota-Twin Cities. Graduate supervisor: Jenny Filson. Faculty advisor: Dr. Mark Snyder.

August 2006-August 2008: **Undergraduate research assistant**, Economics department, South Dakota State University. Faculty advisor: Dr. Scott Fausti

TEACHING EXPERIENCE:

- Associate Lecturer, University of Wisconsin-Milwaukee
  - Introduction to Conditioning and Learning (online) – Spring 2015, Fall 2015
- Teacher Assistant, University of Wisconsin-Milwaukee, Fall 2010 to Spring 2015.
  - Physiological Psychology, discussion – Fall 2010, Spring 2011, Fall 2011
  - Advanced Physiological Psychology, lab – Spring 2012, Fall 2012, Fall 2013, Spring 2014, Fall 2014, Spring 2015
  - Introduction to Psychological Statistics, discussion and lab – Spring 2013

POSITIONS HELD:

- Laboratory Manager, University of Wisconsin-Milwaukee, 2012 to current.
- Laboratory Safety Coordinator, University of Wisconsin-Milwaukee, Fall 2015 to current
- Graduate Research Assistantship, University of Wisconsin-Milwaukee, Fall 2015 to current.
- President, Graduate Students in Behavioral Neuroscience, Fall 2014-current
- Vice President, Graduate Students in Behavioral Neuroscience, Spring 2010-Spring 2014
- Treasurer, Psi Chi, Spring 2008-Spring 2010

MEMBERSHIPS:

- Society for Neuroscience, Student member, 2011-current
- Molecular and Cellular Cognition Society, 2011-current
- Pavlovian Society, 2011, 2015
- Association of Graduate Students in Psychology, 2010-current
- Graduate Students in Behavioral Neuroscience, 2010-current
- Psi Chi National Honor Society in Psychology, 2008-current